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Rapid analysis of a plasmid by hydrophobic-interaction chromatography with a non-porous resin

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Abstract

A HPLC technique has been developed, based on hydrophobic-interaction Chromatography with a non-porous packing (TSKgel Butyl-NPR, Tosoh Biosep LLC), that allows separation of the open circular (nicked) and supercoiled forms of five DNA plasmids, ranging in size from 4 to 30 kilo base pairs (kb). The identity of the bands was determined through light scattering and gel electrophoresis. Several buffers, gradients, flow-rates and temperatures were evaluated in determining the optimum operating conditions for the separation. For all plasmids a reversed ammonium sulfate in phosphate buffer (pH 7.1) gradient was established. The chromatographic resolution between the supercoiled and nicked peaks was found to be a function of flow-rate and temperature. The resolution and the elution order did not vary with plasmid size, with the open-circular form always being eluted before the supercoiled form. Hydrophobic-interaction chromatography is a useful alternative to ion exchange or size exclusion for the chromatography of large plasmids, up to 30 kb.

Keywords: Hydrophobic-interaction chromatography; DNA

1. Introduction

There is a growing interest in the use of non-viral vectors such as DNA plasmids in vaccine and gene therapy applications because of the safety concerns associated with viral vectors. DNA plasmids are extra-chromosomal closed circular double stranded DNA found most frequently but not exclusively in bacterial cells. They replicate independently of the cell chromosome, and typically carry genes that encode for antibiotic resistance, toxin production, and breakdown of natural products. There are three possible forms of a plasmid not including aggregates (concatamers): supercoiled, open circular and linear. The supercoiled plasmid is a circular double stranded DNA that coils helically on itself to form a super helix. The supercoiled form exhibits maximal biological activity, and usually travels the fastest on an agarose gel because of its compact shape. If the supercoiled form becomes nicked in one DNA strand, it uncoils to become the open circular or relaxed plasmid form. Because of its large hydrodynamic diameter the open circular (or nicked) form will normally travel slower than the supercoiled form on a gel. If either form is cleaved at a base pair so that it can no longer maintain a circle it becomes the linear form.

Since industrial researchers are developing methods to produce and purify plasmids in large quan-

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tities [1] it is important that quick, reliable, accurate, and inexpensive analytical methods be developed in support of these production processes. Regulatory requirements will mandate that the product plasmid DNA be in a single and precisely defined and characterized isoform. Analytical methods to determine the relative amounts of the supercoiled (or the putative active) and open circular forms of a plasmid have previously been developed using electrophoresis and HPLC. Gel electrophoresis is used routinely to identify the presence of the various forms of a given plasmid [3], with the supercoiled form almost always having the fastest migration rate. Gel electrophoresis of DNA plasmids of modest size (<50 kilo base pairs, kb) is performed with agarose concentrations of 0.5–1%, while larger DNA plasmids have been separated by pulsed gel electrophoresis [2]. Supercoiled DNA ladders are now available to verify the molecular mass of the supercoiled plasmid. Although many researchers have quantified DNA plasmids by the imaging of stained gels [3], more sensitive, linear and rapid quantification techniques continue to be pursued [4].

The separation of plasmid DNA forms using high-performance liquid chromatography (HPLC) has been accomplished using several chromatographic mechanisms, namely; anion exchange [5–7] and gel filtration [8]. Merion and Warren [5] used an anion-exchange column to separate the supercoiled and relaxed forms of PBR322 (4.3 kb) using a linear NaCl gradient over a 30-min run. Prazares [8] effectively separated the supercoiled, nicked and linear forms of a 4.8-kb plasmid using a Sephacryl S1000 SF column. One recent study [9] documents the separation of the supercoiled and open-circular forms of the PBR322 (4.3 kb) plasmid using hydrophobic-interaction chromatography (HIC). Two columns in series were used. The first column used a butyl ligand and the second used an octyl ligand. The columns were loaded in 0.1 *M* sodium phosphate buffer (pH 7.0) containing 2 *M* ammonium sulfate and 1 m*M* EDTA, and eluted with the same solution minus the ammonium sulfate. Quantification of the plasmids eluting from the column is typically performed using UV detection at 260 nm. One recent study indicates that not only the concentration (i.e. amount), but also size (effective radius) and molecular mass of plasmid DNA eluting from chromatography columns can be determined using light scattering [10].

In this research, an analytical HPLC technique based on HIC with non-porous packing was developed for separating the supercoiled and open circular forms of plasmids ranging in size from 4 to 30 kb. The identity of the bands was determined through light scattering and gel electrophoresis. The effect of different salts, flow-rate, temperature and plasmid size on chromatographic resolution was then determined.

2. Experimental

2.1. The plasmids

Two of the five plasmids, a 4.3-kb PBR322 plasmid (PN D9893) and a 9.6-kb PVL Baculovirus Transfer Vector (PN E8647), were purchased from Sigma. From gel electrophoresis of these plasmids, it was evident that most of the plasmid was in the supercoiled form, however nicked and linear plasmid forms were identified by the presence of two lighter bands with slower gel migration rates.

The three larger plasmids (PQR186, PQR150 and PMT 103) were obtained from the Department of Molecular Biology at University College London [4]. PQR 186 and PQR150 were 13 and 20 kb, respectively, and both carried a resistance gene for kanamycin. PMT103 is lower copy number 29 kb plasmid, and carries a resistance gene for ampicillin. Over half of the DNA sequence in these three larger plasmids is homologous, allowing for comparison of the chromatogaphic separation efficiency based solely on plasmid size. All three plasmids had been transformed into *Escherichia coli* DH5α. The cells containing the plasmid were stored at −70 °C until the time of use. One ampoule (1 ml) was used to inoculate 500 ml of LB broth. The 2-l flask agitated in a shaker cabinet at 250 RPM and 37 °C for 15 h. The kanamycin- and ampicillin-resistant cells were grown in the presence of 10 μg/ml of kanamycin and 200 μg/ml ampicillin (Sigma), respectively. At harvest, 10 ml of the cell suspension was centrifuged. The resulting pellet was resuspended in 500 μl of 1× TE buffer, and processed through a standard Qiagen DNA prep (QIAprep spin Miniprep,

No. 27106). Ultimately, 75 μl of plasmid in TE buffer was collected per sample. Gel electrophoresis results indicate that in the 13- and 20-kb samples, all three plasmid forms were present in near-equal proportions. Before injecting onto the Butyl-NPR column, these samples were concentrated by approximately 10× using Microcon-50 (Millipore) membranes.

2.2. Gel electrophoresis

The agarose concentration (0.5–0.75%) and the run times (7–18 h) were adjusted, depending on the size of the plasmid. All gels were run in TAE buffer at 47 V. For example, the 20- and 29-kb plasmid ran on a 0.5% agarose gel for 18 h. A supercoiled DNA ladder (Gibco, No. 15622) was used for size confirmation, and a High DNA mass ladder (Invitrogen, No. 10496-016) was used to confirm a linear relationship between mass of DNA in a given band and band intensity. The gels were stained with Syber green I (BioWhitaker, No. 50512), imaged (Fluor-Imager 595, Molecular Dynamics Inc.) and then analyzed with the Image-Quant software (Molecular Dynamics).

2.3. HPLC

A TSKgel Butyl-NPR column (PN 14947, Tosoh Biosep LLC) was used to perform the HPLC separation for each plasmid evaluated. The column utilizes HIC to accomplish the separation. The hydrophobic ligand is a butyl group, attached to a non-porous methacrylate backbone. Therefore, the stationary phase is stable in a pH range of 2–12. The particles are 2.5 μm in diameter, packed into a 3.5 cm ×4.6-mm I.D. stainless steel column (column volume: 0.58 ml).

The TSKgel Butyl-NPR column was installed on an Agilent 1100 series HPLC system. This includes a quaternary pump, vacuum degasser, autosampler, thermostated column compartment, and UV–Vis diode-array detector with a micro flow cell (6 mm flow path).

For the purposes of identifying the chromatographic peaks, fractions were collected for gel electrophoresis. For fraction collection up to 15 μl of a 0.5-μg/μl solution of the two smaller, purchased plasmids (PBR 322 and pVL) were loaded onto the column. For the three larger plasmids obtained through shaker flask *E. coli* fermentations and subsequent DNA preparations, significantly less plasmid DNA was loaded onto the column. For a given injection, up to 20 μl of 0.05 μg/μl plasmid solution was loaded onto the column. The column was equilibrated in sodium phosphate buffer (50 m*M*) pH 7.1 containing 3 *M* ammonium sulfate and eluted with a 10 column volume linear gradient from 3 to 0 *M* ammonium sulfate ammonium sulfate to determine the best separation for each size plasmid. The larger plasmids were more hydrophobic and could be loaded in as low as 2.4 *M* ammonium sulfate and still bind to the stationary phase.

The effect of lyotropic salts, such as sodium citrate (1.5 *M*), sodium chloride (3 *M*), sodium sulfate (2.5 *M*), and ammonium sulfate (3 *M*) dissolved in phosphate buffer (50 m*M*) pH 7.1 were investigated. The 9.6-kb pVL plasmid was dissolved in sodium phosphate buffer (50 m*M*) pH 7.1 containing 1.5 *M* ammonium sulfate prior to loading onto the TSKgel Butyl-NPR HPLC column. A 10 column volume (11.6 min at 0.5 ml/min) reverse linear salt gradient was used from the starting salt concentration listed to no salt in phosphate buffer (50 m*M*) pH 7.1.

For the purposes of understanding the effect of flow-rate and temperature on the separation, the 9.6-kb pVL plasmid was dissolved in sodium phosphate buffer (50 m*M*) pH 7.1 containing 1.5 *M* ammonium sulfate prior to loading onto the TSKgel Butyl-NPR HPLC column. Flow-rates from 0.5 to 1 ml/min and temperatures from 25 to 55 °C were also scouted with a 10 column volume (11.6 min) linear gradient from 3 to 1.5 *M* ammonium sulfate in 50 m*M* sodium phosphate pH 7.1.

The effect of plasmid size on resolution was determined with 4.3-, 9.6- and 20-kb plasmids. Each of the plasmids was subjected to a 10 column volume (11.6 min) linear gradient from 3 to 1.2 *M* ammonium sulfate in 50 m*M* sodium phosphate pH 7.1 with a 0.5-ml/min flow-rate at 25 °C.

2.4. Topoisomerase treatment

To verify the location of the open circular form of the plasmid in the chromatogram, 13 μg of the

9.6 kB pVL plasmid was treated with 20 units of topoisomerase II (PN 73590Y, United States Biochemical Corp.). The reaction mixture was incubated for 15 min at 30 °C with 13 μl H_2O, 1.3 μl of 10× Topoisomerase II Reaction Buffer which contains 100 m*M* Tris–HCl, pH 7.9–500 m*M* NaCl–500 m*M* KCl–50 m*M* $MgCl_2$–1 m*M* EDTA–150 μg/ml bovine serum albumin (BSA)–10 m*M* ATP. The reaction was stopped with 1.5 μl of 7 m*M* EDTA. This gave a final concentration of 0.37 μg/μl of pVL plasmid. For comparison, 1 μl of 0.37 μg of the plasmid before and after topoisomerase treatment as well as an enzyme blank was injected on the TSKgel Butyl-NPR column. A 10 column volume (11.6 min) linear gradient was run from 3 to 1.2 *M* ammonium sulfate in 50 m*M* sodium phosphate pH 7.1 with a 0.5-ml/min flow-rate at 25 °C.

2.5. Light scattering

A multi angle light scattering (MALS) detector from Wyatt Technology Corporation (Model DAWN EOS) was also added after a UV detector (260 nm) to measure molar mass and root-mean-square (RMS) radius of the forms of the 9.6-kb plasmid eluted from the TSKgel Butyl-NPR column.

3. Results

3.1. Identification of chromatographic peaks for the 9.6-kb PVL plasmid

The majority of the HPLC experiments in this research focused on the separation of the various forms of the 9.6-kb pVL Baculovirus Transfer vector. Fig. 1 shows a chromatogram obtained by using a 10 column volume linear gradient from 3 to 1.5 *M* Ammonium sulfate in 20 m*M* Tris pH 8 at 0.5 ml/min and at 25 °C with the Butyl-NPR column. The top chromatogram is from a 90° LS detector and the bottom is from a UV detector at 260 nm. Both

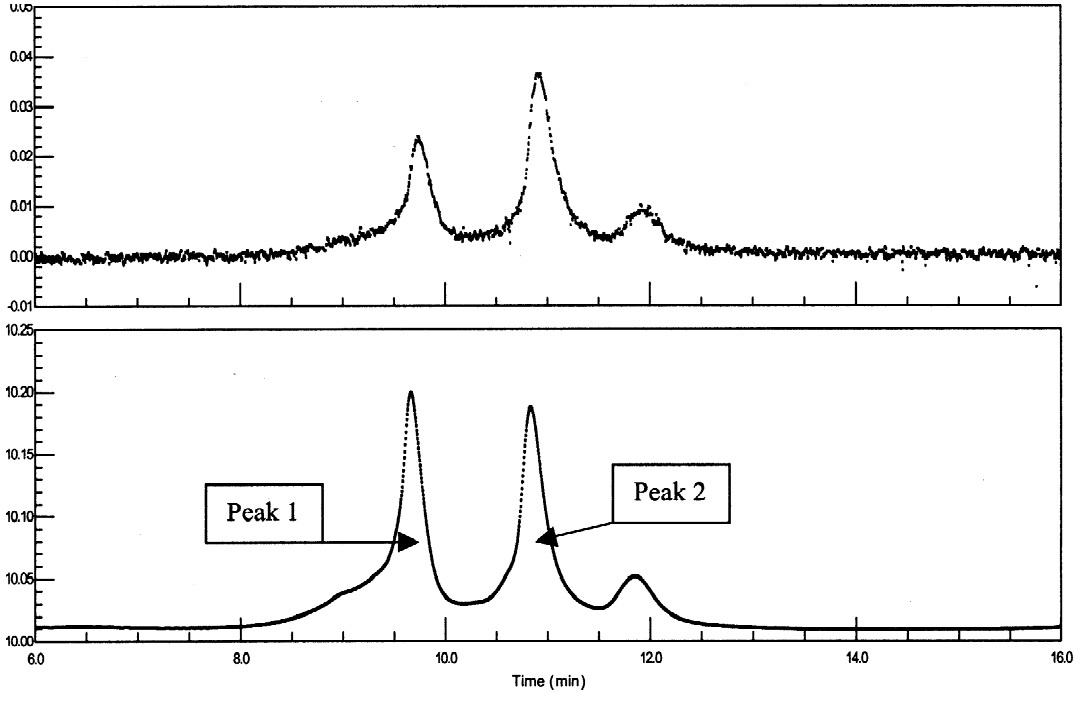

Fig. 1. Chromatograms of the 9.6-kb PVL 1392 Baculovirus transfer vector. The top chromatogram is from a 90° LS detector and the bottom is from a UV detector at 260 nm. Run conditions: 10-column volume linear gradient from 3 to 1.5 *M* ammonium sulfate in 20 m*M* Tris pH 8 at 0.5 ml/min and at 25 °C.

chromatograms show three primary peaks. Gel electrophoresis of this same sample had indicated the presence of all three plasmid forms, along with some high-molecular-mass DNA. From the gel image, it was clear that the supercoiled plasmid was highest in concentration, followed by the open circular. Only trace amounts of the linear form were detected on the gel. Since three chromatographic peaks were detected following elution from the Butyl-NPR column, each with a A_{260}/A_{280} ratio of approximately 1.8 (indicative of DNA) it seemed reasonable to presuppose that the three DNA plasmid forms eluted in the following order: open circular, supercoiled and linear. To verify the identity of the peaks, several approaches were taken.

3.1.1. Light scattering analysis

Based on light scattering theory [11], the amount of light scattered at zero scattering angle is directly proportional to the product of the polymer molecular mass, the specific refractive index increment (dn/dc), and the concentration. The angular variation of the scattered light is associated with the RMS radius of the molecule. The dn/dc for DNA in aqueous buffer was found to be 0.17 ml/g in the literature [12]. The DNA concentration at each eluting slice was calculated from the UV absorbance at 260 nm using the extinction coefficient of 20 for a 1-mg/ml solution and a 1-cm path length. The scattered light intensity from DAWN EOS at each eluting slice was then plotted as a function of different angles (15–165° in this case) as shown by a typical Debye plot in Fig. 2. From Debye plots for each of the three chromatographic peaks, the molecular mass and RMS radius of each of the three eluted plasmid forms were determined. The first two DNA molecules that were eluted had the same molecular mass, while the third (last) one eluted had approximately double that molecular mass. The molecular mass determined in this manner was approximately one-half that expected for a 9.6-kb plasmid. The difference is likely due to the effect of salt on the extinction coefficient.

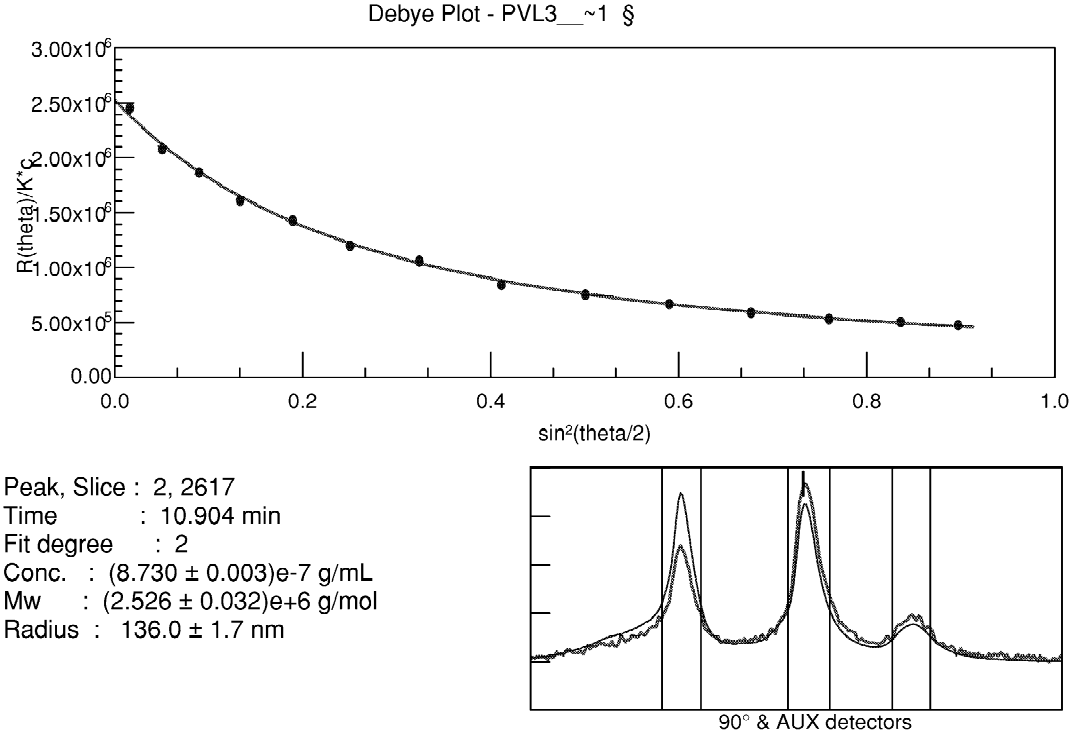

Fig. 2. Debye plot (top) of one data slice from the chromatogram (bottom right). Molecular mass and RMS radius are obtained from the respective intercept and slope of this plot.

The DNA molecule corresponding to the second eluted peak was smallest in radius (135 nm), followed by the first eluted DNA (175 nm) and the last one eluted (185 nm). This light scattering data supports the likelihood that the second eluted peak is supercoiled pVL plasmid since it would be the most compact. The first peak to elute appears to be either nicked or linear plasmid, or a combination of both. The third peak to elute may be a concatameric form of the 9.6-kb plasmid DNA because it has a molecular mass that is double that of peaks 1 and 2.

3.1.2. Topoisomerase treatment

A 13-μg amount of the 9.6 kB pVL plasmid was treated with 20 units of topoisomerase II. Topoisomerase II alters the topological state of DNA by passing the helix through a transient break. This relaxes the supercoiled to the open circular form of the plasmid. For comparison, 1 μl of 0.37 μg of the plasmid before and after topoisomerase treatment as well as an enzyme blank was injected on the TSKgel Butyl-NPR column. From Fig. 3, it is clear that before topoisomerase treatment there is very little peak 1, with most of the plasmid in peak 2 or the supercoiled form. After topoisomerase treatment however, most of peak 2 is converted to peak 1. Therefore, peak 1 contains the vast majority of the open circular DNA present in the sample of 9.6 kb plasmid DNA. Peak 2 contains the vast majority of supercoiled plasmid. The third peak is only evident if a larger amount (0.5 μg) of the pVL plasmid is loaded onto the column (shown on the top chromatogram of Fig. 3), and does not contain significant amounts of either the supercoiled or nicked forms of the plasmid.

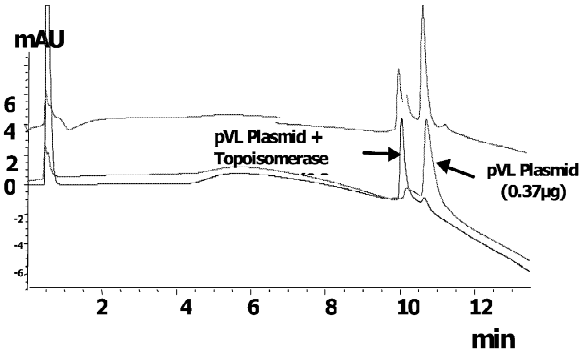

Fig. 3. pVL plasmid was treated with with topoisomerase II. For comparison, 1 μl of 0.37 μg of the plasmid before and after topoisomerase treatment was injected on the TSKgel Butyl-NPR column.The third eluting peak is discernible at a higher injection level (0.5 μg) of plasmid (without topoisomerase), as shown by the top chromatogram.

3.1.3. Gel electrophoresis

As final confirmation of the peak identities, fractions were collected from a 15-μl injection of 0.5 μg/μl PVL sample on the Butyl-NPR column. The fractions were run on an 0.5% agarose gel, which was subsequently stained with Syber green I to detect DNA and imaged. The results (Fig. 4) indicate that peak (or fraction) 2 contains almost all supercoiled plasmid, with only a trace amount of linear and open circular (nicked) plasmids. The band corresponding to the linear form of the 9.6-kb plasmid from peak 2 migrated a distance which is identical to that migrated by the 10-kb linear DNA segment present in the DNA ladder mixture, confirming the molecular mass of the DNA in the column eluent. The first eluted peak is composed entirely of nicked pVL plasmid. These gel results are consistent with the light scattering and topoisomerase data. The first peak is primarily nicked plasmid and the second peak is primarily supercoiled plasmid. From the gel result, however, it is apparent that the this HIC technique effectively separates the supercoiled and nicked plasmid forms, but may not separate the supercoiled and linear plasmid forms as well.

Chromatographic data for all five plasmids evaluated in this research looked similar, in that the three primary peaks appeared to correspond to the three plasmid forms. The gel results for the three larger plasmids (13, 20 and 29 kb) however, are similar to that presented in Fig. 4. The first peak is primarily nicked plasmid and the second peak is primarily supercoiled plasmid. The identity of the third peak (not shown in Fig. 4) is assumed to be concatameric DNA, based on the estimated molecular mass from light scattering data. Fraction analysis for these plasmids indicates that the elution order does not change with plasmid size (4–30 kb). Since the separation mechanism (with these non-porous beads) is based solely on hydrophobic interaction between the plasmid DNA and the butyl ligands attached to the beads, the elution order observed in these experi-

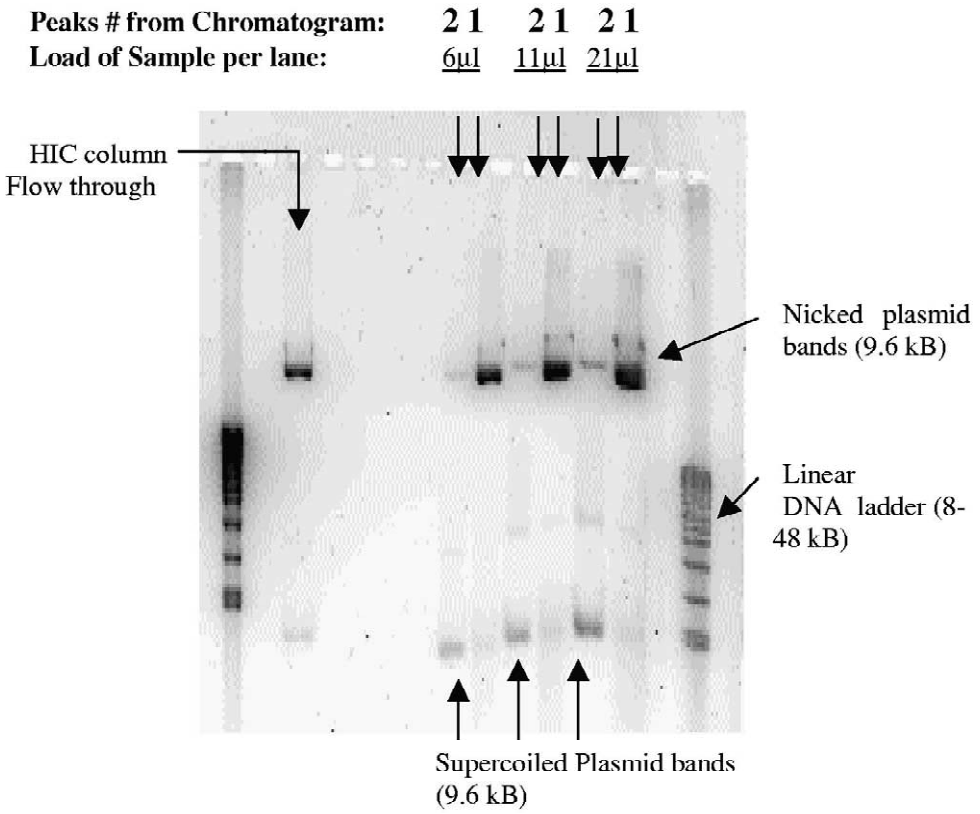

Fig. 4. A 0.5% agarose gel, stained with Syber Green, of fractions of the pVL 1392 plasmid that were collected from a 15-µg injection on the Butyl-NPR column.

ments suggests that the nicked form of plasmid has an exposed surface that is less dense in hydrophobic patches than that of the supercoiled form.

3.2. Factors effecting resolution

A range of salts, flow-rates and temperatures were evaluated in determining the optimum operating conditions for the separation plasmids in the size range of 4–30 kb.

3.2.1. Salt type

Various lyotropic salts, such as 1.5 *M* sodium citrate, 3 *M* sodium chloride, 2.5 *M* sodium sulfate and 3 *M* ammonium sulfate in phosphate buffer (50 m*M*) pH 7.1 were evaluated as potential eluents with the Butyl-NPR column. The 9.6-kb pVL plasmid did not bind to the stationary phase with the 1.5 *M* sodium citrate (which is it's solubility limit) or 3 *M* sodium chloride and came out in the void volume of the column (Fig. 5). The 2.5 *M* sodium sulfate precipitated in the reservoir over the course of the experiment, so no further work was done with it. The five plasmids were retained on the column in 3 *M* ammonium sulfate in 50 m*M* sodium phosphate pH 7.1. In fact, there was an insignificant amount of plasmid in the flow through fractions for all five plasmids. Gel runs with all five plasmids indicate that all of the plasmid forms are stable in ammonium sulfate concentrations $\leq$3 M.

3.2.2. The effect of flow-rate and temperature on resolution

The effect of flow-rate and temperature on the resolution of the open circular and the supercoiled form of the 9.6-kb plasmid is presented in Fig. 6. Flow-rate has a more pronounced effect on resolution than temperature. As the flow-rate decreases

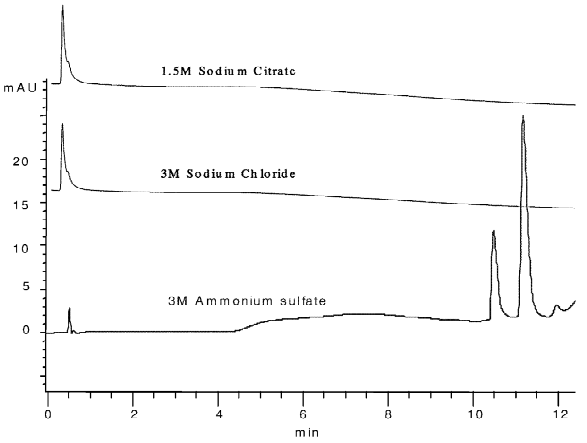

Fig. 5. Evaluation of different salts as eluents.

from 1 to 0.5 ml/min the resolution improves from 31 to 52% depending on the temperature. The reason for this is that the band widths of both peaks decrease as the flow-rate decreases. It is not surprising that slower flow-rates reduce the widths of the eluting DNA peaks, since DNA is such a large molecule which would therefore diffuse slowly from the particle into the buffer during elution. Interestingly, at 55 °C the resolution at 1 and 0.5 ml/min is the same. Increasing the temperature from 25 to 45 °C increases the resolution by 38% (from 1.6 to 2.4) at 1 ml/min and by 26% at 0.5 ml/min. As shown in Fig. 7, the elution volume between the two peaks increases at higher temperatures, resulting in the calculated resolution increase. Furthermore, the elution times for both the supercoiled (peak 2) and open-circular (peak 1) plasmid forms decrease as the temperature increases. These results indicate that the temperature increase allows for both plasmid forms to be eluted into a higher salt environment, with the open-circular form exhibiting this tendency more so than the supercoiled form. There is however a significant downside to operating at temperatures >25 °C: the supercoiled plasmid form is converted to the open circular form during the HIC step. Therefore, these results indicate that any analytical assay developed around this HIC technique be performed at 25 °C, so that an estimate of the ratio of the quantities of the various forms of the plasmids could be obtained. The magnification of the chromatograms in Fig. 7 is too low in this figure to see the 3rd peak. Separation of the three primary peaks was evident for all conditions evaluated, with the required separation time being only 7–12 min.

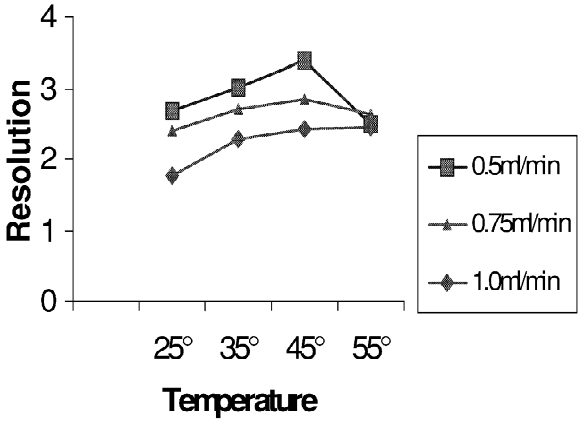

Fig. 6. Chromatographic resolution of the supercoiled (peak 2) and nicked (peak 1) forms of the 9.6-kb PVL plasmid versus flow-rate and temperature.

3.2.3. The effect of plasmid size on resolution

The effect of plasmid size on resolution with a 4.3-, 9.6- and 20-kb plasmid is presented in Fig. 8. Each of the plasmids was subjected to the same 10 column volume linear gradient from 3 to 1.2 *M* ammonium sulfate in 50 m*M* sodium phosphate pH 7.1 at 0.5 ml/min. The results indicate that the resolution between the open circular and supercoiled forms is not a function of plasmid size. The separation between the supercoiled form and the third peak does however improve as the size of the plasmid increases.

4. Discussion

An orthogonal analytical technique for analysis of plasmids has been developed. While ion exchange separates based on charge and size exclusion based on size, this HIC method separates based on hydrophobicity. This method has been demonstrated to separate the supercoiled and open-circular forms of plasmids, ranging in size from 4 to 30 kb. Due to the predominantly hydrophillic nature of DNA, high salt concentration (i.e. 3 *M* ammonium sulfate) was

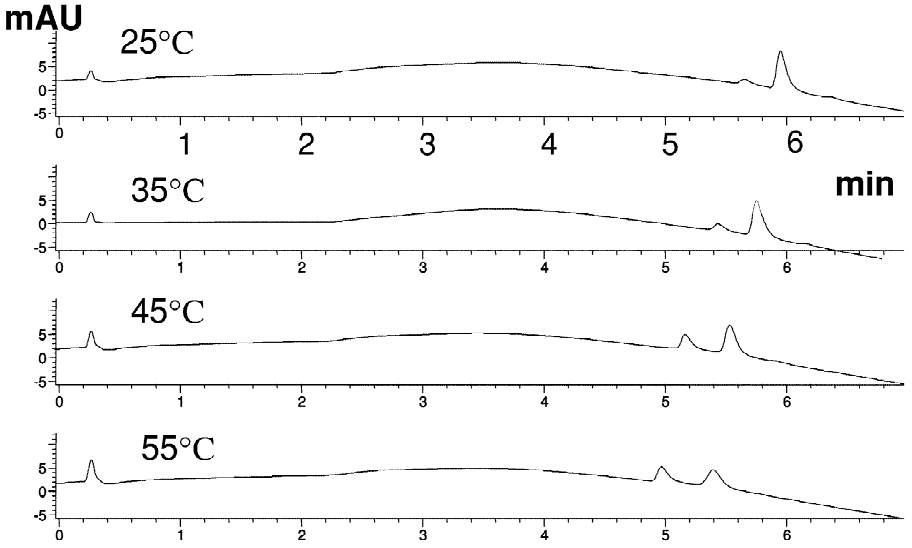

Fig. 7. The 1-ml/min series (used in Fig. 4), showing the effect of temperature from 25 to 55 °C on the peak size. A 10-column volume linear gradient from 3 to 1.5 *M* ammonium sulfate in 50 m*M* PO_4 buffer pH 7.1 was employed. It shows that as the temperature increases, the resolution and degradation (thermal) of open circular to supercoiled forms of the 9.6-kb plasmid both increase.

required to bind the plasmid DNA onto the resin surface (covered with butyl groups). The results from this work indicate that the open-circular form of the plasmid elutes before the supercoiled form, in a reverse salt gradient. One possible explanation for this is that the supercoiled form of the plasmid is more hydrophobic than the open circular form possibly due to its compactness and concentration of hydrophobic patches in a smaller area. This explanation seems reasonable since the underwinding of supercoiled DNA makes it easier to separate (partially denature) DNA strands, exposing the more hyrdophobic (base pairs) parts of the DNA molecule. If the third peak is an aggregate of the plasmid it follows that it would be the most hydrophobic form. It was also observed that the larger the plasmid the less salt needed to bind it to the Butyl-NPR column. Larger plasmids also are eluted later using the same gradient indicating an increase in hydrophobicity (data not shown).

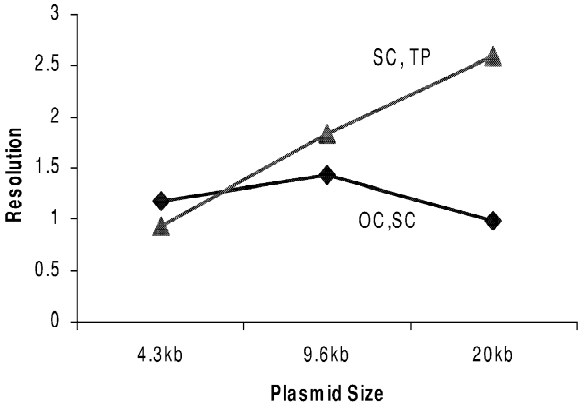

Fig. 8. Resolution versus plasmid size (at 25 °C and 0.5 ml/min), for peak 2 (supercoiled or SC) vs. peak 1 (open circular or OC), and for peak 2 (supercoiled) vs. peak 3 (TP).

Additionally this technique is faster than size exclusion. As plasmid quality control becomes more routine, and higher throughput is required, this becomes very important. This is an advantage of the nonporous stationary phase of the Butyl-NPR column, which has the added benefit of eliminating the problem of finding a resin with a pore size large enough for a 30-kb plasmid. Scale-up of this technique would be difficult however because of the low capacity (surface area) of the nonporous beads and the high pressure drop that results from a column being packed with these very small (2.5 μm) beads.

5. Conclusion

Based on this research, HIC is a useful alternative to anion exchange or electrophoresis, for the separation of the plasmid forms. The Butyl-NPR column provides a high resolution, fast HPLC method. The forms of a plasmid can be analyzed in under 7 min at 1 ml/min for plasmids up to 30 kb. The chromatographic resolution between the supercoiled and nicked peaks was found to be a function of flow-rate and temperature but not plasmid size.

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