Supercoiled Circular DNA Retention in Nonequilibrium Chromatography: Viscosity and Velocity Dependence—Behavior Difference with Proteins

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Abstract

This study demonstrates that the retention behavior of various circular double-stranded DNA molecules (3, 5, and 10 kb) increases over the entire flow-rate range (0.02–1.8 mL/min) at all the mobile phase viscosities (h). The transition between the two well-known nonequilibrium chromatography methods (slalom and hydrodynamic chromatography) is clearly visualized for proteins and does not appear for plasmids because of their strong compact structure. Also, the optimal conditions for F and h are determined to obtain the most efficient separation of these three plasmids in a minimum analysis time.

Introduction

The separation of biomolecules by conventional high-performance liquid chromatographic (HPLC) modes is usually based on an equilibrium phenomenon between mobile and stationary phases. Alternative chromatographic procedures are available for biological and synthetic polymers and are based on the use of a laminar flow, which occurs in the three-dimensional network of pores created in the column packing (the spherical particle filling the column) (1–12). One of these analytical methods, which is called hydrodynamic chromatography (HDC), has been principally developed and applied to the separation of synthetic polymers such as polystyrenes (4–6). The elution order in HDC is the same as in gel permeation chromatography (GPC) because of the exclusion of the large polymers from the low velocity regions near the spherical particle wall (4) (Figure 1). The smallest polymers are eluted after the larger ones (Figure 1). The macromolecules can be thus separated in packed columns on the basis of the effective radius of the random coil polymer. The separation in HDC occurs when the ratio between the polymer radius and the pore radius (dependent on the spherical particle diameter filling the column) is between 0.01 and 0.35. Guillaume et al. (13) recently demonstrated the connection between the HDC and GPC processes. Another chromatographic procedure based on hydrodynamic phenomena was recently developed and is called slalom chromatography (SC). This analytical method has been reported for the separation of linear double-stranded DNA fragments (6–11). In SC, the linear DNA fragment progressed through the closed column packing like a snake edges its way into long grass (Figure 2). The elution order for the linear DNA fragment is the opposite of that expected for an HDC or GPC mechanism, the larger strands are eluted after the smaller ones (6) (Figure 2). When the DNA chain is applied to a chromatographic system, the chain frequently goes around the spherical obstacles; the larger the fragments, the more difficulty it has travelling across the interstitial spaces created inside the column. Guillaume et al. also demonstrated that the SC and HDC modes are interconnected and that the HDC↔SC transition existed and can be clearly visualized for the DNA fragments (14). GPC, HDC, and SC modes can be linked in a global separation mechanism based on a nonequi-

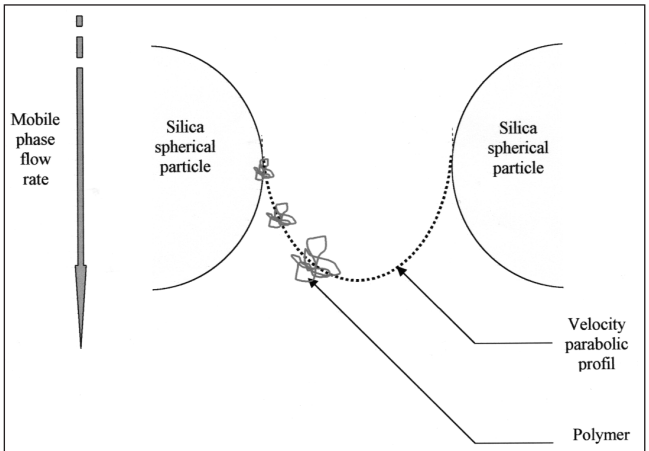

Figure 1. Representation of the polymer migration in hydrodynamic chromatography through the closed-column packing particles.

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librium chromatographic principle. Recently, this HDC↔SC transition was also clearly visualized for a series of proteins that possess a certain degree of compactness (15). It appeared necessary to test the existence of the HDC↔SC transition for macromolecules that are less flexible than the proteins, like supercoiled circular DNA. This one is a circular double-stranded DNA that coils helically on itself to form a super helix with a strong compact shape. In this paper, the migration of various circular DNA fragments was analyzed in nonequilibrium chromatography over a wide range of flow rates and mobile phase viscosities, and their elastic properties were compared with those obtained for proteins.

Experimental

Apparatus

The HPLC system consisted of a Merck Hitachi pump L7100 (Nogent-Sur-Marne, France), an Interchim Rheodyne injector model 7125 (Montluçon, France) fitted with a 20-µL sample loop, and a Merck L4500 diode array detector. A Chromasil column (2-µm particle size, 50- × 4.6-mm column size) and C1 (total carbon content including that used for silicon coating, 4.5%) supplied by Interchim was used with a controlled temperature at 25°C in a TMN°701 Interchim oven. An RM 180 Rheostat temperature stabilized rotary viscosimeter (Rheometric Scientific, Caluire, France) was used to measure the viscosity of each mobile phase.

Reagents

Circular double-stranded DNA (P_3, P_5, and P_{10}), sodium hydrogen phosphate, and sodium dihydrogen phosphate were purchased from Prolabo (Paris, France). Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse-osmosis cartridge.

Chromatographic conditions

The mobile phase consisted of a 0.001M sodium phosphate solution at pH 6.5 with different mass fractions of glycerol in order to modify the mobile phase viscosity. The following 10 glycerol fractions were included in this range: 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, and 0.10. Twenty microliters of plasmid solution was injected, and the retention time was measured for different flow-rate (F) values.

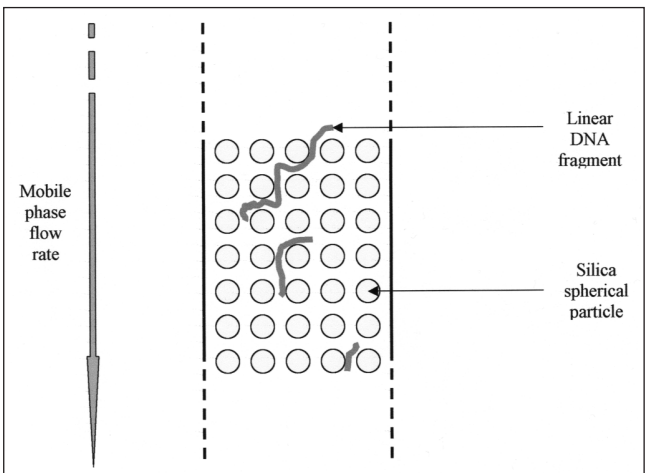

Figure 2. Representation of the linear DNA fragment reptation in slalom chromatography through the closed-column packing particles.

Mobile phase flow-rate studies

Compound relative retention times (RRT) were determined over the flow rate of 0.020–1.800 (± 0.001) mL/min. The chromatographic system was left to equilibrate at each flow rate for at least 30 min before each experiment. To study this equilibration, the retention time of the P_3 plasmid was measured after 30 min and 1 and 2 h. The maximum relative difference between the retention times of this compound was never more than 0.5%, which meant that after 30 min the chromatographic system was sufficiently equilibrated for use.

Additionally, at low a flow rate, no significant effect of pressure transients during the injection were detected with the system employed for these measurements.

Results and Discussion

In nonequilibrium chromatography, DNA retardation under different conditions was compared in terms of RRT as defined by t_R/t_0 (10,15) where t_R is the DNA retention time and t_0 corresponds to the void fraction of the column. Experimentally, t_0 was determined by a triplicate injection of 1 µL of potassium iodide

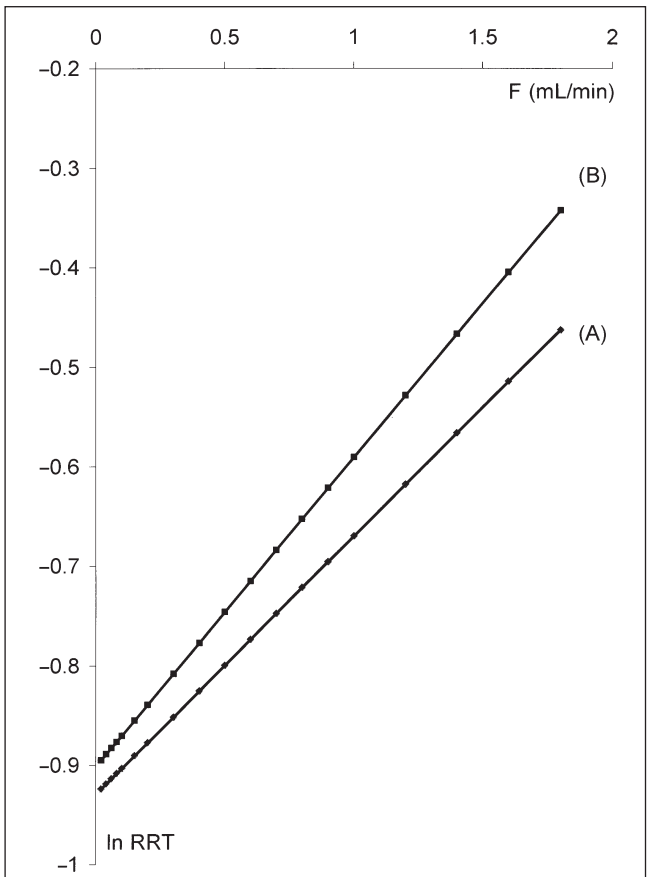

Figure 3. The plasmid RRT in relation to the mobile phase F value (A) without glycerol (h = 0.92) and (B) for a glycerol fraction equal to 0.10 (h = 1.17).

(KI). Hirabayashi and Kasai (10) have previously shown that columns developed for reversed-phase chromatography (such as the C1 column used in this study) are useful for SC. In order to eliminate a possible hydrophobic interaction that could interfere with the hydrodynamic principle, an aqueous mobile phase containing 5–20% of organic modifier (such as acetonitrile) was used (10). It was found that the hydrophobic interaction was negligible in such conditions. Thus, to specifically study the glycerol effect on mobile phase viscosity, the experiments were carried out with a proportion of 5% in the mobile phase. The fact that there was no significant difference in the void time t_0 values at a constant flow rate for various glycerol fraction concentrations was consistent with a hydrodynamic mechanism. Also, an undesirable hydrophobic interaction is characterized by poor recovery of the DNA fragments (10). Recoveries of the plasmids were calculated from the chromatographic peak areas. The plasmid recovery (15,16) at a constant flow rate was globally identical (difference ± 7%), whatever the value of the glycerol fraction in the eluent. Thus, it can be concluded that the glycerol effect on the RRT values of the plasmids was the result of the change in the mobile phase viscosity. Also, this showed that the destabilizing effect of polyol on biopolymer related to its capacity to interact with the polynucleotide solvation sites (17) did not significantly affect the plasmid integrity over the glycerol concentration range.

From the t_R and t_0 values, the experimental RRTs were calculated for the different chromatographic conditions. All the experiments were repeated three times. The variation coefficients of the RRT values were less than 0.02 in most cases, which indicated a high reproducibility and good stability for the chromatographic system.

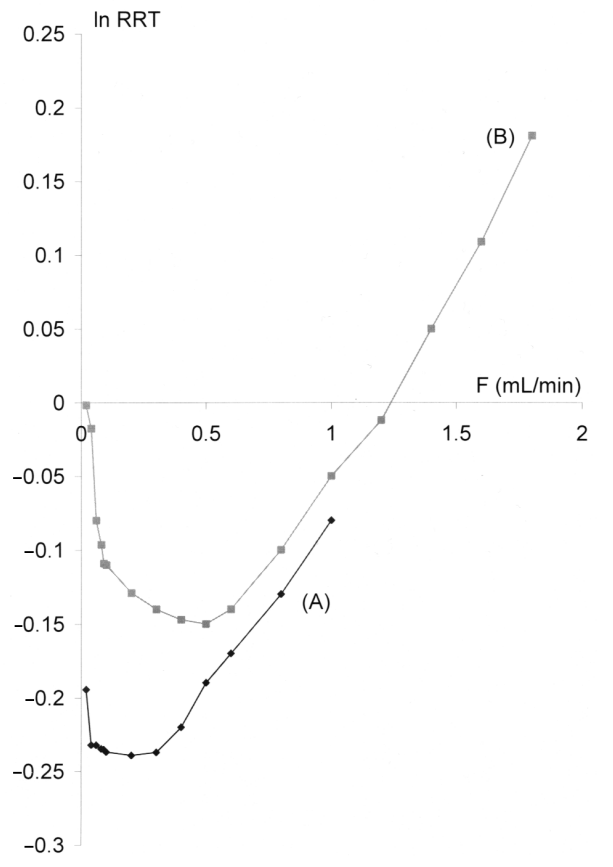

Figure 4. The human serum albumin RRT in relation to the mobile phase F value (A) without glycerol (h = 0.92) and (B) for a glycerol fraction equal to 0.10 (h = 1.17).

The RRT values were plotted against F values for a given plasmid and for the different values of h. For example, Figure 3 shows the variation in ln RRT to F for P_{10} for h = 0.92 and h = 1.17. In each case, the plasmid retention increased over the entire flow-rate and viscosity range. A linear ln RRT versus F behavior was observed; (ln RRT = 0.26F – 0.93 and r^2 = 0.99 for h = 0.92). The retention dependence on the flow rate was different from the one observed for a series of proteins (human, chicken, and bovine serum albumin). Indeed, the protein retention curve showed a decrease in ln RRT until a critical F value (F_c) followed by an increase in the F values (Figure 4) (15). Also, the plasmids were retained less than the proteins (Figures 3 and 4). These differences were attributed to the difference in the compactness between proteins and plasmids.

With the nonequilibrium chromatography technique, the macromolecule retention in a packed column is based on its effective radius. The entire flow rate and glycerol range (ln RRT < 0) confirmed the compact structure of the circular DNA fragment. When F or h increased, the plasmid stretching caused by the shear deformation led to a decrease in the size transverse in relation to the flow direction, which made the effective radius of the plasmid decrease. Thus, the plasmids tended to go to the lowest velocity regions near the particle wall, and the RRT values increased (hydrodynamic retention behavior). This behavior was confirmed by the fact that at all of the mobile phase flow rates or mobile phase viscosity values, the retention order of the plasmids throughout the column was the same as in GPC (i.e., they increased when 3 > 5 > 10 kb). The larger the circular DNA (P_{10}), the more it was excluded from the low velocity regions near the particle wall. Contrary to the protein retention (Figure 4), ln RRT was < 0 in the entire flow-rate range. Thus, for plasmids that had a higher compact structure than the proteins, the HDC→SC transition was not observed.

An example of separation optimization of the three plasmids was studied. The quality of the separation of the mixture of plasmids was assessed by means of the following response function:

$$\delta = \min(\alpha_{app}) \text{ if } \min(\alpha_{app}) \leq \alpha_l \qquad \text{Eq. 1}$$

$$\delta = \alpha_l + \frac{1}{t_{R(P3)}} \text{ if } \min(\alpha_{app}) \leq \alpha_l \qquad \text{Eq. 2}$$

where α_{app} is the apparent selectivity between two adjacent plasmids on the chromatogram that is defined as the ratio of their RRT, $\min(\alpha_{app})$ is the value of the apparent selectivity for the worst separated pair of peaks on the chromatogram, α_l is called the apparent limit selectivity because it is the minimum value of the worst selectivity accepted for the separation, and $t_{R(P3)}$ is the analysis time [i.e., the retention time of the last (P_3) fragment on the chromatogram]. Therefore, if the resolution for the worst separated pair of peaks on the chromatogram was lower than the chosen extreme selectivity, the δ function would have been equal to the selectivity. If not, separation conditions were obtained and then the analysis time intervened in the form of $1/t_{R(P3)}$. Thus, the

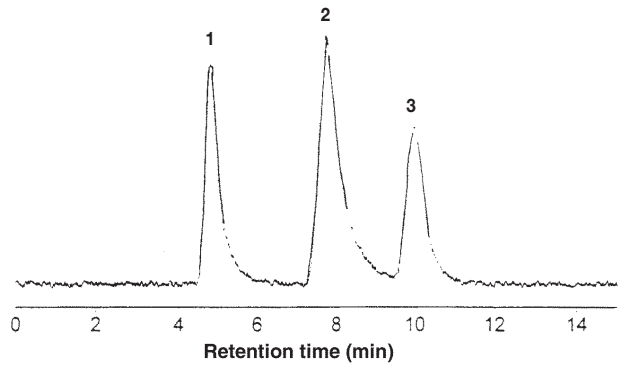

Figure 5. Chromatography of the three circular DNA fragments: (1) 10 kb, (2) 5 kb, and (3) 3 kb. Experimental data using a C1 Chromasil column with a particle diameter = 2 µm for a mobile phase flow rate = 0.05 mL/min and a mobile phase viscosity = 1.02.

δ function was maximal when both efficient separation conditions and a minimum analysis time were obtained. Using a weighted nonlinear regression procedure (18–20), both RRT values of each plasmid and $t_{R(P3)}$ can be modelled by a second order polynomial in relation to F and h:

$$\ln RRT \text{ (or } \ln t_{R(P3)}) = a_0 + a_1F + a_2h + a_{12}Fh + a_{11}F^2 + a_{22}h^2 \quad \text{Eq. 3}$$

where a_0, a_1, a_2, a_{12}, a_{11}, and a_{22} are constants. The correlation coefficients for the fits were higher than 0.999. Knowing the variation of the apparent selectivity values of the plasmids and the analysis time with F and h, the δ values can be given for different values of the two factors. The extreme selectivity was equal to 1.5. Using a pure random search (21), δ reached its maximum because mobile phase viscosity and velocity values were equal to 1.02 and 0.050 mL/min, respectively. The corresponding chromatogram is given in Figure 5.

Conclusion

In this study, a thesis was developed to show that the strong compact structure of plasmids explained the retention behavior difference between plasmids and proteins in nonequilibrium chromatography. The beginning of the SC mode (i.e., the HDC ↔ SC transition) clearly visualized for the proteins did not appear for very low flexible molecules such as plasmids. Additionally, the optimal F and h values were determined to obtain the best separation of the plasmids with a minimum analysis time.

References

1. E.A. Marzio and C.M. Guttmann. Separation by flow. *Macromolecules* **2:** 131–46 (1970).
2. H.J. Small. Hydrodynamic chromatography: a technique for size analysis of colloidal particles. *J. Colloid. Interface. Sci.* **48:** 147–54 (1974).
3. D.A. Hoagland and R.K. Prudhomme. Hydrodynamic chromatography as a probe of polymer dynamics during flow through porous media. *Macromolecules* **22:** 775–81 (1989).
4. G. Stegeman, J.C. Kraak, and H. Poppe. Hydrodynamic and size-exclusion chromatography of polymers on porous particles. *J. Chromatogr. A* **550:** 721–39 (1991).
5. G. Stegeman, J.C. Kraak, and R. Tijssen. Hydrodynamic chromatography of polymers in packed columns. *J. Chromatogr. A* **657:** 283–93 (1993).
6. E. Venema, J.C. Kraak, H. Poppe, and R.J. Tijssen. Polymer characterization using on-line coupling of thermal field-flow fractionation and hydrodynamic chromatography. *J. Chromatogr. A* **765:** 135–44 (1997).
7. J. Hirabayashi and K. Kasai. *Molecular Interactions in Bioseparations.* T.T. Ngo, Ed. Plenium Press, New York, NY, 1993, p. 69.
8. E. Boyes, D.G. Walker, and P.L. McGeer. DNA separation by slalom chromatography. *Anal. Biochem.* **170:** 127–34 (1988).
9. J. Hirabayashi, N. Ito, K. Noguchi, and K. Kasai. Slalom chromatography: size-dependent separation of DNA molecules by a hydrodynamic phenomenon. *Biochemistry* **29:** 9515–21 (1990).
10. J. Hirabayashi and K.I. Kasai. Improved DNA separation by the use of columns developed for reverse-phase chromatography. *J. Chromatogr. A* **722:** 135–42 (1996).
11. E. Peyrin, Y.C. Guillaume, C. Grosset, A. Ravel, A. Villet, J. Alary, and A. Favier. Mobile-phase-viscosity dependence on DNA separation in slalom chromatography *J. Chromatogr. A* **886:** 1–12 (2000).
12. Q.H. Wan. Effect of electrical double-layer overlap on the electroosmotic flow in packed-capillary columns. *Anal. Chem.* **69:** 361–63 (1997).
13. Y.C. Guillaume, J.F. Robert, and C. Guinchard. A mathematical model for hydrodynamic and size exclusion chromatography of polymers on porous particles. *Anal. Chem.* **73:** 3054–64 (2001).
14. Y.C. Guillaume and C. Guinchard. Peculiarities of the retention mechanism of circular and linear DNA fragment using non-equilibrium chromatography. *J. Liq. Chromatogr and Relat. Technol.* **24:** 1061–72 (2001).
15. Y.C. Guillaume, M. Thomassin, J.F. Robert, and C. Guinchard. Mobile phase viscosity and velocity dependence on protein retention using nonequilibrium chromatographic techniques. *J. Chromatogr. Sci.* **39:** 361–64 (2001).
16. J. Hirabayashi and K.I. Kasai. Reinforcement of frontal chromatography for effective analysis of lectin-oligosaccharide interactions. *J. Chromatogr. A* **890:** 563–67 (1999).
17. P. del Vecchio, D. Esposito, L. Ricchi, and G. Barone. The effect of polyols on the thermal stability of Calf thymus DNA. *Int. J. Biol. Macromol.* **24:** 361–69 (1999).
18. E. Peyrin, Y.C. Guillaume, and C. Guinchard. HSA-solute interactions, enantioselectivity and binding site geometrical characteristics. *Anal. Chem.* **70:** 4235–40 (1998).
19. E. Peyrin and Y.C. Guillaume. Symmetry breaking during the formation of cyclodextrin-imidazole inclusion compounds: capillary electrophoresis study. *Anal. Chem.* **71:** 2046–2052 (1999).
20. P.R. Bevington. Data reduction and error analysis for the physical sciences. McGraw-Hill, New York, NY, 1969.
21. Y.C. Guillaume and E. Peyrin. Optimizing mobile phase composition, its flow-rate and column temperature in HPLC using taboo search. *Talanta* **51:** 579–86 (2000).

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