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Short communication

Supercoiled circular DNA and protein retention in non-equilibrium chromatography
Temperature and velocity dependence: testimony of a transition

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Abstract

Non-equilibrium chromatography (NEC) is a chromatographic mode for the rapid separation of polymers. The retention behavior of various proteins (human, chicken, bovine serum albumin) and supercoiled circular double-stranded DNA (plasmids) was investigated using a phosphate buffer as a mobile phase at different velocities and column temperatures with a C_1 column with very low-packing particle diameter as a stationary phase. It was shown that the two factors (temperature and velocity) constituted important parameters in the retention mechanism of plasmids and proteins in NEC. The protein was retained more than the plasmid. At all the temperatures (5, 10, 15, 20, 25 °C) the plasmid retention increased over the entire flow-rate range (0.02–1.8 ml/min). For the protein, the retention curve presented a decrease in the relative retention time until a critical value of the mobile phase flow-rate, followed by an increase. The transition between the two well known NEC methods, slalom chromatography and hydrodynamic chromatography was clearly visualized for proteins at the lowest temperature, but did not appear for plasmids due to their strong compact structure.

Keywords: Non-equilibrium chromatography; Flow-rate; Temperature effects; DNA; Proteins

1. Introduction

The separation of biomolecules by conventional high-performance liquid chromatography (HPLC) modes is usually based on an equilibrium between mobile and stationary phases. Alternative chromatographic procedures are available for biological and synthetic polymers. These two techniques, slalom chromatography (SC) and hydrodynamic chromatography (HDC), are based on the use of the laminar flow which occurs in the interstitial spaces created

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between the particles in the column [1–14]. The separation process depends on the flow-rate and the particle size of the column packing and not on the pore size or chemical nature. HDC has been principally developed for and applied to the separation of synthetic polymers such as polystyrenes [4,7,8]. The elution order in HDC is the same as in gel permeation chromatography (GPC) due to the exclusion of the large polymers from the low-velocity regions near the particle wall [4]. Macromolecules can be separated in packed columns on the basis of the effective radius of the random coil polymer. The separation in HDC occurs when the λ ratio between the polymer radius and the channel radius (dependent on the particle diameter) is between 0.01 and 0.35. Guillaume et al. [15] have recently demonstrated the connection between the HDC and GPC processes. Separation in SC has been reported for double-stranded DNA molecules [8–13]. The elution order of the DNA molecules is the opposite of that expected for a HDC or GPC mechanism, the larger strands are eluted after the smaller ones [8]. When the DNA chain is applied to a chromatographic system, it frequently goes around the spherical obstacles; the larger the fragments, the more difficulty it has in travelling across the interstitial spaces created inside the column. It was 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 [16]. GPC, HDC, and SC modes can be linked in a global separation mechanism based on a non-equilibrium chromatographic (NEC) principle. In this paper, the migration of various proteins and circular DNA fragments was analyzed on a C_1 stationary phase over a wide range of flow-rates and column temperatures and their elastic properties were compared.

2. Experimental

2.1. Apparatus

The HPLC system consisted of a Merck–Hitachi pump L7100 (Nogent-Sur-Marne, France) an Interchim Rheodyne injection Model 7125 (Montluçon, France) fitted with a 20 μl sample loop, and a Merck L4500 diode array detector. A C_1 Kromasil column (2 μm particle size: 50 mm×4.6 mm column size) supplied by Interchim was used with controlled temperature (temperature range: 5–25 °C) in a TMN°701 Interchim oven.

2.2. Reagents

Human serum albumin (HSA), bovine serum albumin (BSA), and chicken serum albumin (CSA) were supplied by Sigma–Aldrich (Paris, France). Circular double-stranded DNA (P_3, P_5, P_{10}), sodium hydrogenphosphate and sodium dihydrogenphosphate 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.

2.3. Chromatographic conditions

The mobile phase consisted of a 0.001 *M* sodium phosphate salt at pH 6.5. A 20-μl volume of plasmid solution was injected and the retention times measured for different flow-rate (F) values varying from 0.02 to 1.8 ml/min.

2.4. Temperature studies

Compound relative retention times (RRTs) were determined over the temperature range 5–25 °C. The chromatographic system was left to equilibrate at each temperature for at least 1 h before each experiment. To study this equilibration, the retention times of HSA and P_{10} were measured after 22, 23 and 24 h. The maximum relative difference of the retention time of these compounds was never more than 0.6%, meaning that after 1 h the chromatographic system was sufficiently equilibrated for use. All the solutes were injected three times at each temperature and mobile phase flow-rate.

3. Results and discussion

3.1. Mobile phase velocity dependence on retention in NEC

It has been previously demonstrated that columns developed for reversed-phase chromatography (such

as the C_1 column used in this study) [17] are useful for NEC. In NEC, the RRT of a solute molecule was used as its retention marker [16]. RRT was linked to its retention time t_R and the void time t_0 by RRT= t_R/t_0. t_0 was obtained by a triplicate injection of 1 μl of KI. The asymmetry factor of all peaks calculated from measurements made at 50% of the total peak height was in the range $1.00 \leq A_s \leq 1.10$. From t_R and t_0 values determined at the maxima of the chromatographic peaks, 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 2% in most cases indicating a high reproducibility and good stability for the chromatographic system. The RRT values were plotted against *F* values for a given protein and plasmid and for the different values of *T*. For example, Figs. 1A and 2A show the variation in ln RRT to *F* for HSA and the P_{10} circular DNA form at $T=25$ °C. It can be noted that in all cases, ln RRT was <0 showing that both the protein and the plasmid structures were in a compact form [16]. Nevertheless, the proteins were compacted the lowest and then were, in all cases, retained more than the plasmids confirming a hydrodynamic retention (HDR) behavior [4–6].

For the circular DNA fragment, the retention increased over the entire flow-rate range. With the NEC technique, it is well known that the macromolecule retention in a packed column is based on the effective radius of its compact structure [4–6]. When *F* increased, the plasmid stretching caused by the shear deformation led to a decrease in the size transverse in relation to the flow direction making 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. This HDR behavior was confirmed by the fact that at the lowest mobile phase flow-rate, the elution order of the plasmid through the column was the same as in gel permeation chromatography, i.e., increased as 3>5>10 kilobase pairs (kbp). The larger the circular DNA (P_{10}), the more it was excluded from the low velocity regions near the particle wall.

For the proteins, the retention was divided into two domains, one for *F* values below F_c and a second for values above F_c.

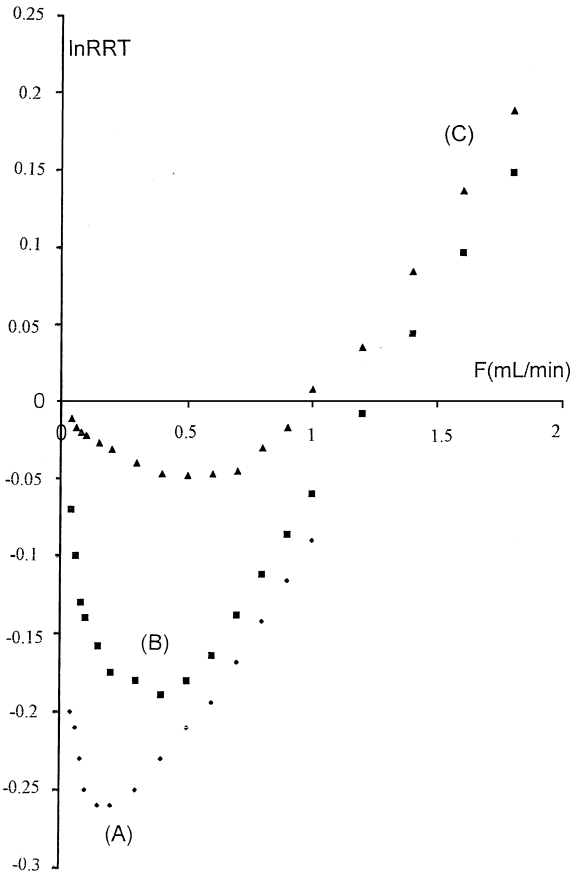

Fig. 1. The HSA RRT in relation to the mobile phase *F* value (A) $T=25$ °C, (B) $T=15$ °C, (C) $T=5$ °C.

(i) For $F>F_c \cong 0.02$ ml/min in a similar way to the plasmids, when *F* increased, the effective radius of the random coil protein decreased producing an increase in the RRT values.

(ii) For $F<F_c$, ln RRT decreased when *F* increased. In this low flow-rate domain, the random coil form of the protein, remained relatively constant in the laminar flow occurring in the interstitial space between the particle packing in the column. When the mobile phase velocity increased, the proteins were excluded from the lowest velocity region near the particle wall producing a decrease in the RRT values also confirming a HDR behavior [4–6].

3.2. Temperature dependence on retention in NEC

For each temperature, the ln RRT versus *F* curve

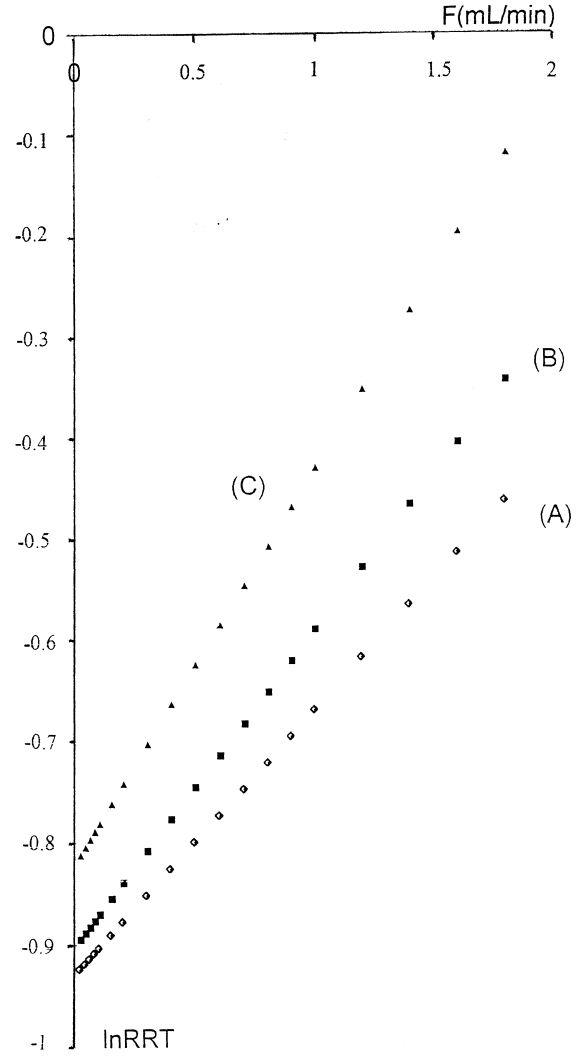

Fig. 2. The plasmid RRT in relation to the mobile phase F value (A) T=25 °C, (B) T=15 °C, (C) T=5 °C.

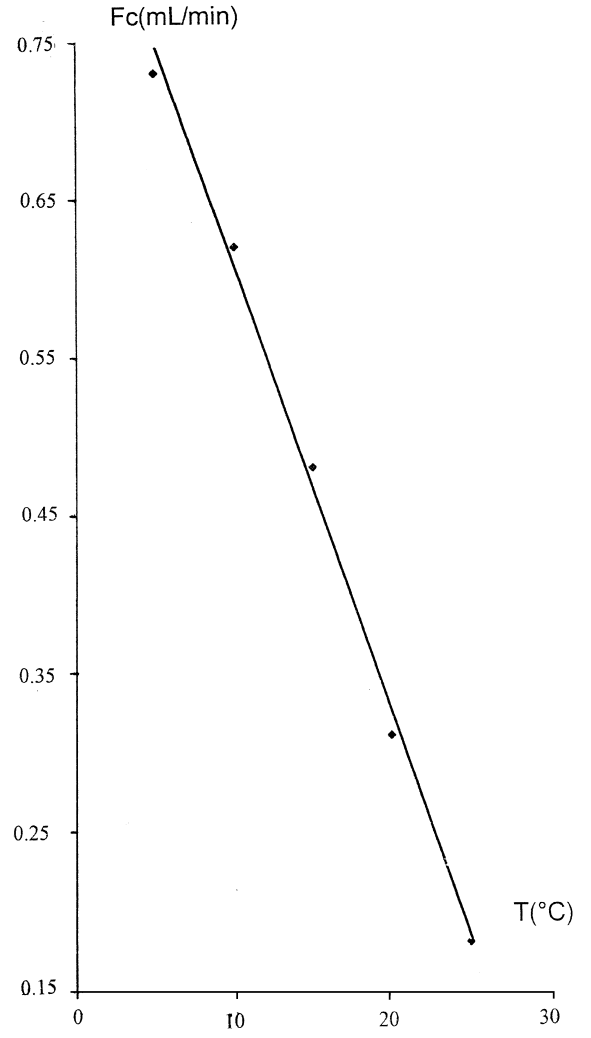

Fig. 3. The plot for F_c versus T.

was similar to the previous one (i.e., the plasmid retention increased over the entire flow-rate range and a critical F_c value was observed for only proteins). Figs. 1 and 2 show the curves for HSA and P_{10} for three different temperatures (5 °C, 15 °C, 25 °C). A similar behavior was observed for the two other proteins and plasmids as well as column temperature.

A linear F_c versus T behavior was observed [for HSA, $F_c=-0.026T+0.844$, $r^2=0.999$ (Fig. 3)]. The values and Figs. 1-3 lead to the following conclusions:

(i) For plasmids and proteins (for $F>F_c$) (Figs. 1 and 2) in which ln RRT<0 the ln RRT values increased as T decreased. This phenomenon can be explained by the dependence of the acetonitrile–water mobile phase viscosity (η) estimated by the use of the empirical relationship reported by Ghrist et al. [18] $\eta=\eta_{25\,°C}\cdot(298/T)^6$ where $\eta_{25\,°C}$ is the viscosity at 25 °C. When T decreased, the hydrodynamic force generated by the mobile phase increased due to this temperature dependence on the

liquid velocity. Therefore, for proteins and plasmids, there was a decrease in the effective radius of the compact structure of the macromolecule. The proteins and plasmids tended to go to the lowest velocity regions near the particle wall and the ln RRT value increased (HDR behavior).

(ii) For the proteins only, the ln RRT can become positive. For example, for HSA and at 15 °C, ln RRT>0 when F was above 1.2 ml/min ($=\hat{F}$) (Fig. 1). In this case, the protein structure being much more flexible than that of plasmid, began to lose its compact structure and migrated through the packing more or less in a curvy fashion (unlike a spherical particle) via the SC mode. For $T=15$ °C in the 0.5–1.8 ml/min range, ln RRT versus F always had a linear behavior (Fig. 1) representing the equation $\ln \mathrm{RRT}=0.26F-0.31$ ($r^2=0.998$, for HSA). The F value in which ln RRT changed its sign ($\hat{F}$) corresponded to the HDC→SC transition. This result confirmed that both the SC and HDC mechanisms can be interconnected for proteins, as was previously demonstrated for more flexible molecule such as linear DNA fragments using the NEC technique. In conclusion, in this paper, it was demonstrated that the strong compact structure of plasmids explained the retention behavior difference between plasmids and proteins in NEC. For each temperature, the protein retention curve there was a decrease in RRT until a critical F value of the flow-rate, followed by an increase, while the plasmid retention increased over the entire flow-rate. As well, the beginning of the SC mode (i.e., the HDC→SC transition) was clearly visualized for the first time only for the less compact biopolymers (i.e., proteins) for low column temperature and particular values of F ($\hat{F}$).

References

[1] L.A. Marzio, C.M. Guttmann, Macromolecules 2 (1970) 131.
[2] H.J. Small, J. Colloid Interface Sci. 48 (1974) 147.
[3] D.A. Hoagland, R.K. Prudhomme, Macromolecules 22 (1989) 775.
[4] G. Stegeman, J.C. Kraak, H. Poppe, J. Chromatogr. 550 (1991) 721.
[5] E. Peyrin, Y.C. Guillaume, A. Villet, A. Ravel, C. Grosset, J. Alary, A. Favier, J. Liq. Chromatogr. Rel. Technol. 24 (2001) 1245.
[6] Y.C. Guillaume, M. Thomassin, C. Guinchard, J. Chromatogr. Sci. 39 (2001) 361.
[7] G. Stegeman, J.C. Kraak, R. Tijssen, J. Chromatogr. A 657 (1993) 283.
[8] E. Venema, J.C. Kraak, H. Poppe, R.J. Tijssen, J. Chromatogr. A 749 (1996) 159.
[9] J. Hirabayashi, K. Kasai, in: T.T. Ngo (Ed.), Molecular Interactions in Bioseparations, Plenum Press, New York, 1993, p. 69, Chapter 5.
[10] E. Boyes, D.G. Walker, P.L. McGeer, Anal. Biochem. 170 (1988) 127.
[11] J. Hirabayashi, N. Ito, K. Noguchi, K. Kasai, Biochemistry 29 (1990) 9515.
[12] K.I. Kasai, J. Chromatogr. 618 (1993) 203.
[13] E. Peyrin, Y.C. Guillaume, C. Grosset, A. Ravel, A. Villet, J. Alary, A. Favier, J. Chromatogr. A 886 (2000) 1.
[14] Q.H. Wan, Anal. Chem. 69 (1997) 361.
[15] Y.C. Guillaume, J.F. Robert, C. Guinchard, Anal. Chem. 73 (2001) 3059.
[16] Y. Guillaume, M. Thomassin, J.F. Robert, C. Guinchard, J. Liq. Chromatogr. Rel. Technol. 24 (2001) 1061.
[17] J. Hirabayashi, K.I. Kasai, J. Chromatogr. A 849 (1999) 563.
[18] B.F. Ghrist, M.A. Stadalius, L.W. Spyder, J. Chromatogr. 387 (1987) 1.