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Mobile-phase-viscosity dependence on DNA separation in slalom chromatography

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Abstract

Slalom chromatography (SC) is an alternative chromatographic procedure for the separation of relatively large double-stranded DNA molecules and is based on a new principle. The retardation of the DNA fragments from the cleavage of the Lambda DNA by the *Kpn*I restriction enzyme was studied using an acetonitrile–phosphate buffer as a mobile phase with various concentrations of viscosity modifier (i.e. glycerol) and a C_1 column as a stationary phase. The DNA molecule retention was accurately described over the glycerol concentration range using a model previously established. It was shown that the eluent viscosity increase enhanced the slalom chromatographic capacity to separate the DNA fragments. A connection between SC and 'hydrodynamic chromatography' processes was predicted to link the two processes in a global separation mechanism based on a non-equilibrium principle.

Keywords: Slalom chromatography; Viscosity; DNA

1. Introduction

The separation of biopolymers such as DNA by conventional HPLC modes is based on an equilibrium phenomenon between mobile and stationary phases. In the case of ion-exchange chromatography, the retention mechanism is dependent on the electrostatic interaction between the phosphate groups of DNA molecules and the cationic groups of the stationary phase. For hydrophobic-interaction chromatography, an additional contribution to the macromolecule retention is encountered via the hydrophobic effect between the stationary phase and DNA bases. Finally, gel permeation chromatography is dominated by the capacity of the DNA molecules to penetrate into the stationary-phase pores in relation to their size. Recently, a new chromatographic mode for the size-dependent DNA fractionation was discovered independently by Boyes et al. [1] and Hirabayashi and Kasai [2]. This slalom chromatography (SC) is based on a completely new separation mechanism. Using columns for size-exclusion chromatography, the elution order is the opposite to that expected for a gel mechanism, the larger strands are eluted after the smaller ones [3]. This separation

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depends on the flow-rate and the particle size of the column packing and not on their pore size or chemical nature [4]. In this case, the column packing is only used for the formation of the network of narrow interstices between spherical particles. When a DNA chain is applied to a chromatographic system, it frequently turns around the spherical obstacles; the larger the fragments or the smaller the particle size, the more difficult it is for the fragments to travel across the interstitial spaces created inside the column. According to this description, this separation mode cannot be explained in terms of an equilibrium constant between the mobile and the stationary phases. Another chromatographic technique, i.e. column hydrodynamic chromatography (HDC), used for the separation of polymers or particles, shows the same characteristic. This technique is also based on the use of a laminar flow which occurs in the interstitial spaces created between the particles packed in the column [5–7]. However, the elution order in HDC is the same as in gel permeation chromatography due to the exclusion of the large polymer from the low velocity regions near the particle wall [8].

The SC procedure can provide a novel research approach to the fractionation of macromolecules. The main advantage of slalom chromatography is constituted by the rapidity and the simplicity of the experimental procedure. However, its resolution ability is inferior to that of capillary gel electrophoresis [9]. Thus, it was of interest to study the possibilities of enhancing the fractionation capacities of this technique. On the basis of a model which has been previously proposed to accurately describe the mechanistic aspect of the separation in slalom chromatography [10], it was expected that the mobile-phase viscosity η was one of the main parameters which could influence DNA separation. In order to gain further insight into the fractionation mechanism and enhance the efficiency of the technique, the retention of DNA fragments on a C_1 stationary phase was analyzed over a wide range of glycerol concentrations (used as a viscosity modifier) and at various linear velocities v (i.e. flow-rates). The retention behavior in SC was integrated in a more general concept which implied the polymer hydrodynamic separation retrieved in HDC.

2. Experimental section

2.1. Apparatus

The HPLC system consisted of a Shimadzu pump LC 10 AT VP (Touzart et Matignon, Courtaboeuf, France), an Interchim Rheodyne injection model 7125 (Montluçon, France) fitted with a 20-μl sample loop and a Merck L 4500 diode array detector. A C_1 Kromasil column (particle size, 5 μm; column size, 150×4.6 mm; pore diameter, 100 Å) supplied by Interchim, was used with controlled temperature at 25°C in an Interchim Crococil oven TM No. 701. A RM 180 Rheomat temperature-stabilized rotary viscosimeter (Rheometric Scientific, Caluire, France) was used to measure the viscosity of each mobile phase.

2.2. Reagents

Lambda DNA [48.50 kilobase pairs (kb)] and restriction enzyme *Kpn*I were supplied by New England Biolabs (Gagny, France). Ethanol, EDTA, acetonitrile, glycerol, 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. Digestion of λ DNA

Restriction enzyme *Kpn*I was used for the cleavage of the λ DNA into three fragments of different sizes: 29.95, 17.05 and 1.50 kb. The λ DNA (2 μg) was treated with 3 U of *Kpn*I in 15 μl of the reaction mixture at 37°C for 3 h, precipitated by ethanol, dissolved in 20 μl of water and stored at −20°C until use.

2.4. Chromatographic conditions

The mobile phase consisted of a 0.01 *M* sodium phosphate salt–0.001 *M* EDTA mixture at pH 6.8 with different concentrations of glycerol varying from 0 to 1 *M*. Twenty μl of DNA solution were

injected and the retention times were measured for different flow-rate values varying from 0.05 to 1.2 ml/min.

3. Results and discussion

3.1. SC using reversed-phase columns

Hirabayashi and Kasai [12] have previously shown that columns developed for reversed-phase chromatography (such as the C_1 column used in this study) are useful for slalom chromatography. In order to eliminate a possible hydrophobic interaction which could interfere with the hydrodynamic principle, they used an aqueous mobile phase containing 5–20% of organic modifier such as acetonitrile [12]. 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 bigger proportion of acetonitrile in the mobile phase (20%). The fact that there was no significant difference in the t_{NR} (the retention time for a non-retained fragment) values at a constant flow-rate for various glycerol concentration was consistent with a 'pure' hydrodynamic mechanism. Also, an undesirable hydrophobic interaction is characterized by poor recovery of the DNA fragments [12]. Recoveries of the DNA fragments were calculated from the chromatographic peak areas. The DNA recovery at constant flow-rate was globally identical (difference $<10\%$) whatever the value of glycerol concentration in the eluent. Thus, it can be concluded that the glycerol effect on relative retention time (RRT) values of the DNA fragments was the result of the change in the mobile-phase viscosity. Also, this showed that the destabilizing effect of polyol on DNA related to its capacity to interact with the polynucleotide solvation sites [13] did not significantly affect the fragment integrity over the glycerol concentration range studied.

3.2. Viscosity dependence on retention and separation in SC

In SC, the separation between two DNA fragments was described by the following relation [4,10]:

$$RRT = t_R / t_{NR}$$

where t_R and t_{NR} are the retention times for a retained and a non-retained fragment (corresponding to the void fraction), respectively. t_{NR} was determined using the 1.5-kb fragment because it is known that the small pore size of 100 Å hinders the permeation of the pores by fragments larger than 100 base pairs (bp) [9]. The retention times for the 1.50-, 17.05- and 29.95-kb fragments (t_R) were obtained at various flow-rate and mobile-phase viscosity. From the t_R and t_{NR} values, the experimental RRT were calculated for the different chromatographic conditions. All the experiments were repeated three times. The RSDs of the RRT values were less than 4% in most cases, indicating a high reproducibility and good stability for the chromatographic system. With a weighted non-linear regression procedure which was used in earlier chromatographic studies [14,15], the data obtained at various flow-rates and viscosity were fitted to the model equations as described previously [10]. The correlation between all the predicted and experimental RRT values exhibited slopes equal to 0.95 with $r^2 > 0.99$. This good correlation between the predicted and experimental values can be considered to be adequate to verify the model. The viscosity dependence on the RRT of the two retained fragments was identical at each eluent flow-rate. For example, Fig. 1 shows the variations of the 17.05-kb fragment in relation to η at various flow-rates. The data are given on the same curve (in relation to the product ηv) in such a way that a wide RRT range is covered. This curve showed a bimodal behavior as previously reported for the 'strict' RRT–flow-rate plots [4,10]. This result confirmed that the hydrodynamic force generated by the fluid in the interparticle channels played a preponderant role in the DNA retention behavior via the molecular stretching as suggested by the experimental [1–4] and theoretical treatments [10]. Concerning the separation between the two retained polymers (17.05 and 29.95 kb), α_{app} corresponding to their RRT ratio was calculated and plotted against ηv (Fig. 2). The separation increased relatively strongly below a ηv product equal to 2.00×10^{-7} Pa m. Above the critical value of this ηv product (ηv_c), a decreasing variation of α_{app} was observed. This

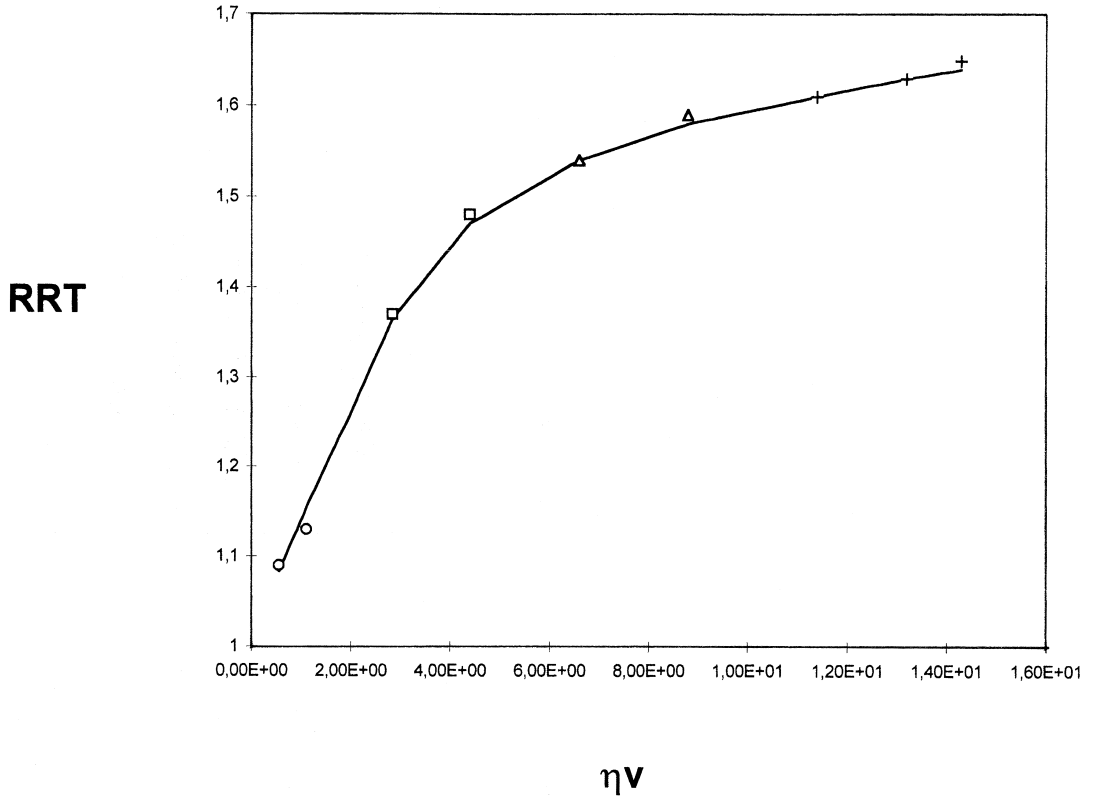

Fig. 1. Theoretical RRT (relative retention time) values calculated using model equations from Ref [10] (—) and experimental RRT values in relation to ηv (10^7 Pa m) for the 17.05-kb fragment. (○) $\eta = 1.1 \times 10^{-3}$ Pa s, flow-rate, 0.05 and 1.1 ml/min; (□) $\eta = 1.1$ and 1.4×10^{-3} Pa s, flow-rate, 0.3 ml/min; (△) $\eta = 1.1$ and 1.4×10^{-3} Pa s, flow-rate, 0.6 ml/min; (+) $\eta = 1.1$, 1.3 and 1.4×10^{-3} Pa s, flow-rate, 1.0 ml/min.

was consistent with the result which has been observed by Boyes et al. [1] concerning the resolution variation with flow-rate for two DNA retained fragments. It was shown from these experiments that the η and v parameters acted in the same manner on DNA retardation (increase in RRT value). The main difference between these two factors was that the viscosity increase was associated with a concomitant enhancement of the analysis time, while the flow-rate increase was associated with the decrease in the analysis time. The higher the liquid velocity, the greater the separation between non-retained and retained molecules and the shorter the analysis time. This fact shows the advantage of the slalom chromatography principle on the equilibrium principle of the classical chromatographic modes. Thus, the optimal conditions for the best separations between the void DNA fraction and other DNA fragments were represented by the highest values of the linear velocity at a constant viscosity which was compatible with a practicable back pressure and the prevention of the physical degradation of DNA fragments. In the case of the separation of the two 17.05- and 29.95-kb large retained fragments, an optimal apparent selectivity was obtained. Fig. 3 shows the chromatogram for the separation of the three fragments analyzed at this optimal apparent selectivity.

3.3. Hypothesis for a connection between SC and HDC mechanisms

As to the SC procedure, the HDC technique is based on the use of the laminar flow occurring in the

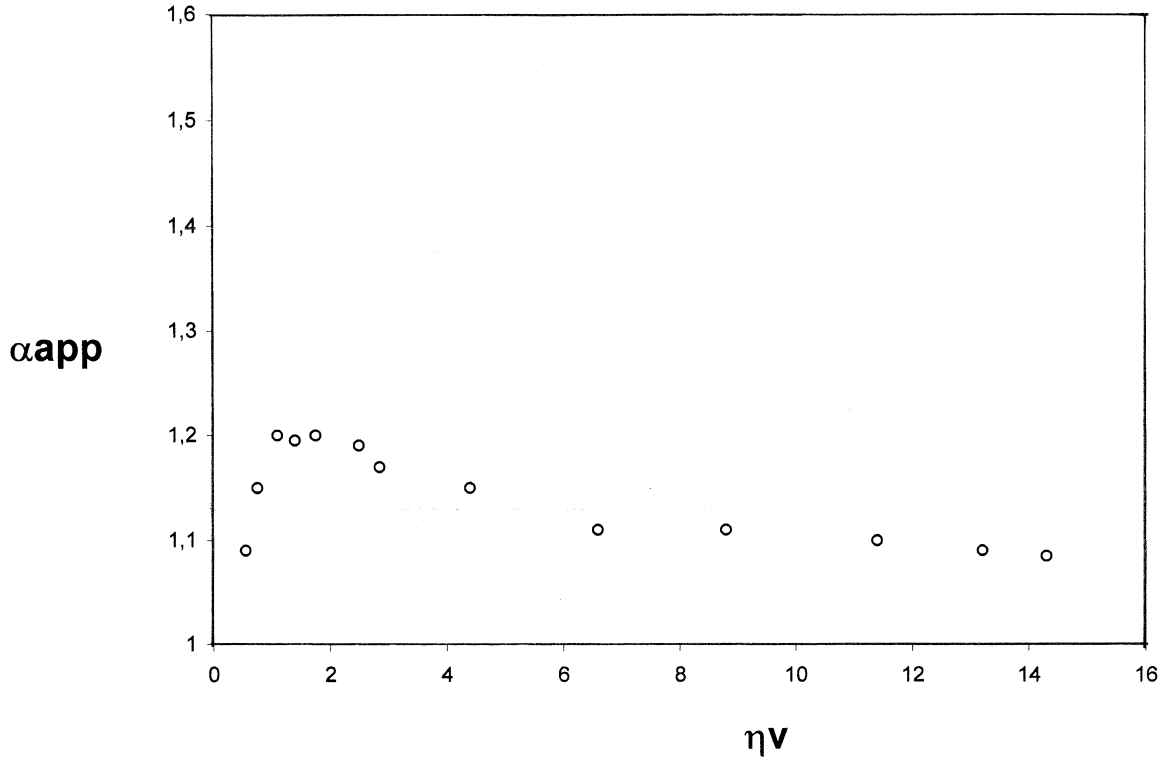

Fig. 2. Experimental apparent selectivity (α_{app}) (○) in relation to ηv (10^7 Pa m) for the two 17.05- and 29.95-kb fragments.

interstitial spaces created between the particles. Synthetic macromolecules can be separated in packed columns on the basis of the effective radius of the random coil polymer r_{eff} [7]. The separation in HDC occurs only when the λ ratio between r_{eff} and the effective channel radius (dependent on the particle diameter) is comprised between 0.01 and 0.35. This λ value is a function of the relative migration parameter τ in such a way that an universal calibration for HDC can be obtained, as shown in Fig. 4a. This migration behavior only occurs when the polymer is in a random coil form. When the mobile-phase velocity increases, the polymer stretching caused by the shear deformation leads to a diminution in the size transverse to the flow direction and then λ decreases [7]. As the polymer deformation is dependent on the Deborah number [8], this flow effect is enhanced when the flow velocity, the viscosity and the molecular mass increase or the particle diameter decrease. Moreover, it has been shown that for a given flow-rate the τ values for large polymers corresponding to a λ value superior to 0.35 are equal and then, no separation occurs [8]. Venema et al. [8] reported that ''this finding might indicate that the large polymers migrate through the packing more or less as a sausage'' (i.e. not as a spherical particle). On the other hand, the separation capacity in SC is strongly dependent on the hydrodynamic force generated by the mobile phase as suggested by the experimental and theoretical results. This is consistent with a retention mechanism which is related to the DNA stretching in the interparticle spaces. Thus, the RRT dependence on the molecular mass in SC shows an optimal selectivity for a critical steady-state extension of DNA which is a function of the diameter particle [10] (Fig. 4b).

On the basis of these two observations, the following hypothesis can be proposed. The separation via the HDC principle occurs when polymers form a random coil configuration. When this configuration is no more observed, due to the decrease in diameter particle or the increase in molecular mass,

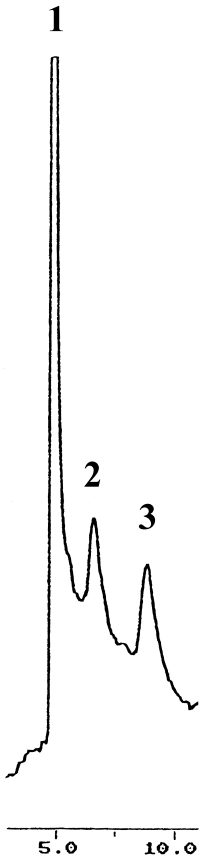

Fig. 3. Chromatograms of the three DNA fragments [1.5 (**1**), 17.05 (**2**) and 29.95 (**3**) kb] at the optimal conditions, time scale in min.

mobile-phase viscosity or flow-rate, the polymer migration in the column remains constant whatever the molecule size. If the molecular mass of the polymer increases again and the extended form produced in the sheared flow is sufficient, it is *expected that the SC principle* can lead to a separation. This hypothesis is presented for the synthetic polymers in Fig. 4a. In the case of DNA molecules, the reverse phenomenon SC→ HDC (when the molecular mass decreases) should also be predicted. However, previous experimental results are not consistent with this expected SC→HDC transition [4]. When the DNA fragments from 0.56 to 9.42 kb are applied on a gel permeation column packed with 9-μm particles, all these excluded fragments are eluted in the same void volume [4]. This implies that

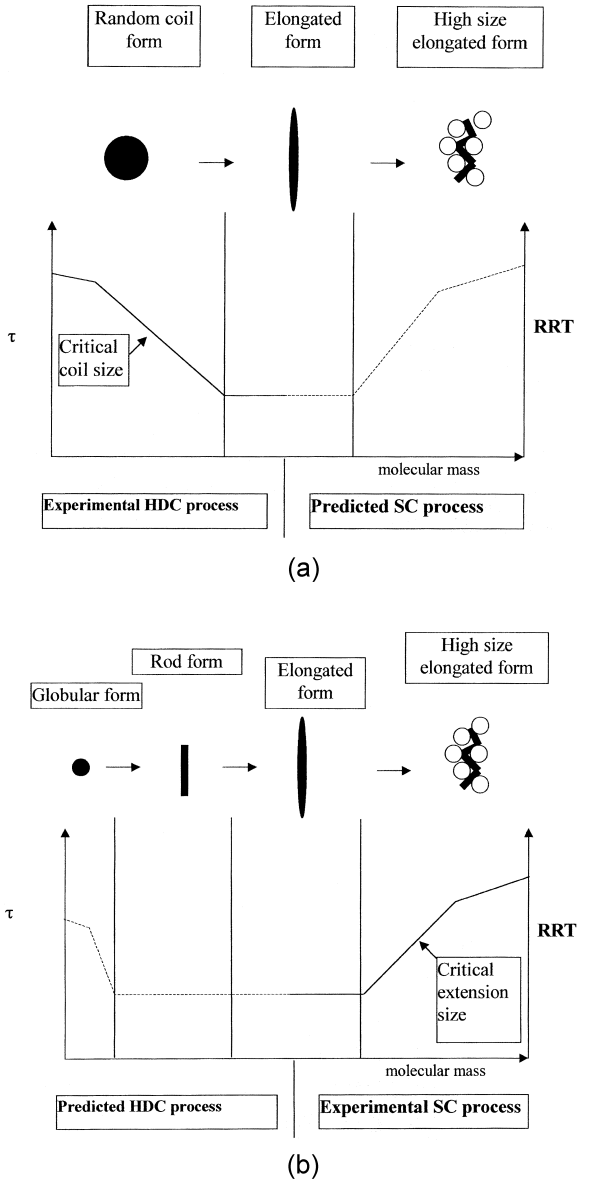

Fig. 4. Schematic representation for a possible connection between SC and HDC mechanisms. (a) For synthetic polymers such as polystyrenes, (b) for DNA polymers.

the HDC principle does not occur. Nevertheless, applying the hydrodynamic theory of the HDC procedure, the λ values of these polymers for this particle diameter are expected to be between 0.01 and 0.35 (using a interparticle porosity of 0.4 and calculating the DNA radius as described by Viovy

and Duke [11]). This fact can be explained by the great shape variability of the double-stranded DNA molecule [9]. For fragments between 20 and 1000 bp, the DNA molecule shape is considered to be as a rod [9,11], and then the HDC mechanism cannot be applied. On the other hand, the DNA fragments larger than 2 kb (2.03, 2.32, 4.36, 6.56 and 9.42 kb [4]) can be treated as flexible random coil [9,11]. However, no allowance is made on a possible deformation of these fragments induced by the hydrodynamic flow in the chromatographic system. The elastic properties of DNA molecules are not the same as for synthetic polymers such as polystyrenes, which are classically separated by the hydrodynamic principle in packed columns. Moreover, in contrast to double-stranded DNA, single-stranded polynucleotides such as RNA exhibit completely different features, i.e. a more compact structure [9]. Thus, it is quite possible that the random coil form of 2–9-kb fragments does not exist under the chromatographic conditions applied in the SC. It can be expected than the HDC mechanism should occur for the $<$20-bp DNA fragments (spherical shape) in packed columns with particles of a very low diameter. Schematic representation of this predictive retention behavior of the DNA molecules in the system SC–HDC is shown in Fig. 4b. In order to assess this hypothesis, systematic analysis of the retention behavior of both synthetic and biological polymers will be carried out in a further work. Different size polymers and columns packed with non-porous particles of a wide range of diameter will be studied.

4. Conclusion

In this paper, it was demonstrated that the main parameter which governed the retention in SC was the hydrodynamic force generated by the mobile-phase flow. This signified that the separation mechanism was strongly dependent on the polymer extension in the interstitial spaces created in the column, as previously reported in the experimental and theoretical approaches. An additional hypothesis was proposed to join the two linear polymer separation mechanisms based on a non-equilibrium principle, i.e. SC and HDC processes, and determine the basis for the expansion of these chromatographic procedures.

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