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Mechanism of DNA Hydrodynamic Separation in Chromatography

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An alternative chromatographic procedure for the separation of large double-stranded DNA molecules was discovered recently and called "slalom chromatography". This fractionation is based on a new hydrodynamic process that is determined by the progression of the mobile-phase flow through the interstitial spaces created between the highly packed particles inside the column. Here, the separation is treated as the result of a slowing down of the large double-stranded DNA fragments in relation to their size with the flow direction changing around the particles. A model, based on the concept derived from the reorientation time of macromolecules, was adequate to describe the hydrodynamic phenomenon. This model constitutes an attractive tool to enhance the expansion of this chromatographic procedure and provide valuable information on the dynamic behavior of biological polymers.

For the analysis and separation of large biological macromolecules such as DNA and RNA, various techniques have been developed over the past few years. The most popular and successful procedure is the (slab or capillary) gel electrophoresis that is based on the theoretical concept of the field dependence on DNA fragment electrophoretic mobility. An alternative technique is high-performance liquid chromatography (HPLC), which is used to separate both small and large molecules. The chromatographic modes for DNA fractionation are gel permeation, ion-exchange, and hydrophobic chromatography. Despite the improvements brought about by the introduction of micropellicular stationary phases,[1,2] chromatographic procedures are considered to be less useful than gel electrophoresis in terms of efficiency and DNA fragment separation range.[3]

However, recently a very promising chromatographic size-dependent DNA fractionation, likely to compete with electrophoretic techniques, has been discovered independently by Boyes and co-workers[4] and Hirabayashi and Kasai.[5] This mode, based on a completely new phenomenon, can separate in a shorter space of time larger double-stranded DNA molecules (ranging from roughly 5 to 50 kb) than the conventional chromatographic procedures by using a column for gel permeation with an ordinary HPLC system. The order of elution is the opposite to that expected for gel permeation chromatography; the larger strands are eluted after the smaller ones. This separation depends on the flow rate and the particle size of the column packing and not on their pore size or chemical nature.[6] The DNA fragments do not interact with the packing matrix because polymer- or silica-based packing have essentially similar separation behavior.[6] Thus, the mechanism of separation cannot be explained in terms of an equilibrium constant between the mobile and stationary phases, but is the result of a hydrodynamic principle. The column packing is used for the construction of very narrow spaces between closely 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 difficulty it has to travel across the interstices created inside the column. Following this description, this separation mode has been called "slalom chromatography".[6] The major disadvantage that limits the expansion of this chromatographic procedure is the fact that the theoretical basis of the separation mechanism has not been elucidated. This report proposes a model, which accurately describes the size dependence of double-stranded DNA molecules.

MODEL

When DNA fragments pass into the column, their direction of progression changes in response to frequent changes in the flow direction through the interstitial spaces. The assumption can be made that fractionation is based on the ability of the DNA chains to adapt to this varying environment. The column packing is treated as a three-dimensional network of pores with an average diameter r. This r diameter is linked to the average particle diameter d_p and the interparticle porosity n by[7]

$$r = 0.42 d_p (n/(n-1)) \qquad (1)$$

The DNA molecule is represented, as in the reptation theory defined by de Gennes,[8] by a chain of P segments, each of contour length r. The sequence of pores followed by the chain is called

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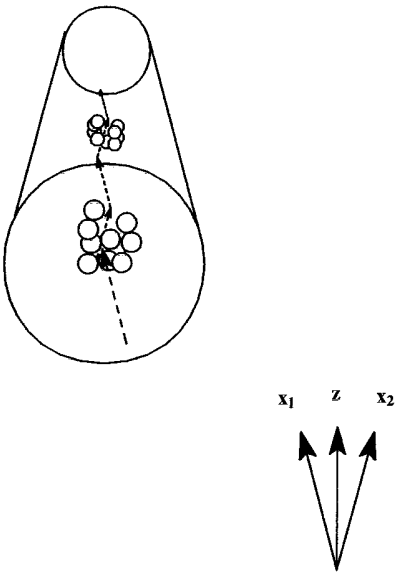

Figure 1. Representation of the progression of the DNA chains (arrows) through the closed column packing particles

the tube with a total length R equal to Pr. The progression of a fragment through the closed column packing can be modeled as shown in Figure 1. There are two cases: (1) When the chain is short, it is aligned in the direction x_1 of the flow before it is switched from an average value of angle θ to the new direction of the flow x_2, and then, the fragment progression is considered as a sequence of independent oriented paths with no or only weak retardation. (2) When the chain is sufficiently large, it is partially aligned along x_1 before the flow turns around the particles going in the x_2 direction and the DNA strand is strongly retarded

This behavior can be analytically treated by introducing the notion of the reorientation time needed for macromolecules as previously reported by several authors for pulsed electrophoresis.[9–11] The reorientation time Γ_o is defined as the time needed for a chain completely aligned along a direction to completely reorient itself and go in a new direction. The larger the DNA molecules, the longer the reorientation time. If J is the curvilinear coordinate along the tube, the reduced curvilinear coordinate j is defined as

$$j = J/Pr \tag{2}$$

When the chain is aligned in the direction x_1 at time $t = 0$, its reduced end-to-end projection along x_1, $k_{X1,}$ is equal to

$$k_{X1}(t{=}0) = K_{X1}/Pr \tag{3}$$

where K_{X1} is the end-to-end distance along x_1. The progression of a DNA fragment is defined as

$$\mathrm{d}j(t) = k_{X1}(t)/\Gamma\ \mathrm{d}t \tag{4}$$

where Γ is the time taken by the chain to travel along the tube length without changing the flow direction. When j varies from 0 to 1, the fraction of j oriented along x_2 is replaced by the same fraction along x_1. On average, the tube is oriented along the diagonal z as reported by Viovy et al.[12] in such a way that the following equation is obtained:

$$k_{X1}\ (0{<}j{<}1) = j + (1 - j)\cos^2(\theta/2) \tag{5}$$

In the reptation theory applied to the gel electrophoresis, the length fluctuations of the chain due to the alternative paths through the pores must be integrated into the models of electrophoretic mobility (biased reptation with fluctuations).[13] The DNA segment that leaves the tube aligns itself preferentially in the field direction to minimize its potential energy. In our chromatography system, there is no difference in the orientation probability for the head segment because the DNA chain follows the flow direction. Combining eqs 4 and 5 gives the following linear differential equation:

$$\mathrm{d}j(t) = \frac{j + (1 - j)\cos^2(\theta/2)}{\Gamma}\,\mathrm{d}t \tag{6}$$

This is solved for j varying from 0 to 1 during time Γ_o by

$$\Gamma_o = -\Gamma(\ln\cos^2(\theta/2))/(1 - \cos^2(\theta/2)) \tag{7}$$

Case 1 described above corresponds to the situation defined by $\Gamma_c > \Gamma_o$, where Γ_c is the average time of the flow direction change along the column. During a time Γ_c, a retarded DNA fragment has the same average velocity as a nonretained very short fragment v_{NR} for the $(\Gamma_c - \Gamma_o)/\Gamma_c$ part of the time and a weaker velocity v during the Γ_o/Γ_c part of the time Γ_c. Thus, the average velocity for a retained molecule v_R is equal to

$$v_R\Gamma_c = v_{NR}(\Gamma_c - \Gamma_o) + v\Gamma_o \tag{8}$$

By definition, $v\Gamma_o = v_{NR}\Gamma$, and then, the retention time t_R of a retained molecule is linked to the retention time of a nonretained one t_{NR} by

$$t_R/t_{NR} = \Gamma_c/(\Gamma_c - (\Gamma_o - \Gamma)) \tag{9}$$

The ratio t_R/t_{NR} is called relative retention time[6] or RRT. If t_p is the average time taken by the flow to cover one pore of the tube, then Γ is equal to Pt_p. In the same manner, $\Gamma_c = P't_p$, where P' is the average number of pores without a change of the flow direction. Therefore, eq 9 is rearranged using eqs 1 and 7 and the equality $P = R/r$ to obtain the RRT for case 1:

$$\mathrm{RRT} = [1 - K(R/d_p)]^{-1} \tag{10}$$

with K constant for a given chromatographic system.

Case 2 is related to the DNA progression when $\Gamma_c < \Gamma_o$. In this situation, the solution of the linear differential eq 6 is

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dependent on Γ_c:

$$j(\Gamma_c) = \frac{\cos^2(\theta/2)}{1 - \cos^2(\theta/2)}(e^{(1-\cos^2\theta/2)\Gamma_c/\Gamma} - 1) \qquad (11)$$

By proceeding as previously described for case 1, the RRT for case 2 is defined by

$$\mathrm{RRT} = \left[K'\frac{R}{d_p}(e^{K''d_p/R} - 1)\right]^{-1} \qquad (12)$$

where K' and K'' are factors only dependent on a given chromatographic system. In eqs 10 and 12, the constants K, K', and K'' are characteristics of the structural arrangement of the spherical particles inside the column and are named directional change factors.

As well, it has been known for many years that DNA molecules have a dynamic behavior in solution.[14] In the absence of an external force, large double-stranded DNA chains are observed to have a random coil state that is entropically the most favorable shape. However, in the presence of an external force (hydrodynamic flow), the DNA chain is extended due to its elastic property.[15] Thus, as the flow rate increases in the chromatographic column, it can be expected that a DNA molecule having a coiled state is gradually stretched. Therefore, the greater the liquid velocity, the larger the increase in the reorientation time around the particles. This is consistent with the experimental flow rate dependence on the relative retention time.[16] Both theoretical and experimental studies[17] have shown that the steady-state extension R of a single molecule in a hydrodynamic flow is characterized by a dumbbell model consisting of two beads connected by a spring representing the entropic elasticity of a wormlike chain. When a force F is applied across the ends of the chain, the extension R is given by[18]

$$\frac{FA}{kT} = \frac{1}{4}\left(1 - \frac{R}{L}\right)^{-2} - \frac{1}{4} + \frac{R}{L} \qquad (13)$$

where A is the persistence length, L the total curvilinear length, k the Boltzmann constant, and T the absolute temperature. In addition, it has been shown by Perkins et al.[19] that the actual bead radius r_b for a chain in an elongational flow is equal to $\alpha L^{0.54}$, where α is a constant. Using the r_b and L values of a single phage DNA molecule calculated from the measurements of its elongation,[17] α can be determined. Knowing the r_b value, the value of F can be obtained with the well-known Stokes relation:

$$F = 6\pi\eta v r_b \qquad (14)$$

where η and v are the mobile-phase viscosity and velocity, respectively.

In our simplified model, the fluid progression along the column is considered homogeneous with a constant velocity in such a way that an analytic approximation for the extension R at a given flow rate can be determined with eq 13 and integrated into the two eqs 10 and 12.

EXPERIMENTAL SECTION

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 L 4500 diode array detector. A porous silica column Zorbax GF250 for gel permeation chromatography (particle size, 4 μm, column size, 250 × 4.6 mm, exclusion size for globular protein, 3×10^5 Da), supplied by Interchim, was used with controlled temperature at 25 °C in an Interchim Crococil oven TM 701.

Reagents. λ DNA (48.50 kb) and restriction enzyme *Kpn*I were supplied by New England Biolabs (Gagny, France). Ethanol, EDTA, 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.

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 units 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.

Chromatographic Conditions. The mobile phase consisted of a sodium phosphate salt 0.01 M−EDTA 0.001M mixture at pH 6.8. A total of 20 μL of DNA solution was injected, and the retention times were measured for different flow rate values varying from 0.03 to 1.2 mL/min. The retention time t_{NR} corresponding to the void fraction was obtained using the 1.5 kb fragment, which was not retained.[4]

RESULTS AND DISCUSSION

Model Validation. The retention time values for the 17.05 and 29.95 kb fragments (t_R) and for the 1.50 kb fragment, which corresponded to the void volume marker (t_{NR}), were obtained at various flow rates to examine the effect of the hydrodynamic flow on the DNA chain retardation. 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 variation coefficients 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 nonlinear regression (WNLIN), which was used in earlier chromatographic studies,[20,21] the data reported by Hirabayashi et al.[16] for four different columns of $d_p = 9$ μm at a flow rate equal to 0.3 and 0.6 mL/min and our data for the Zorbax column of $d_p = 4$ μm at various flow rates were fitted to eqs 10 and 12. The steady-state extension R for the different DNA fragments was calculated from eqs 13 and 14 using, by approximation, the linear velocity of the mobile phase as v value. After the WNLIN procedure, the calculated directional change factors K, K', and K'' characteristic

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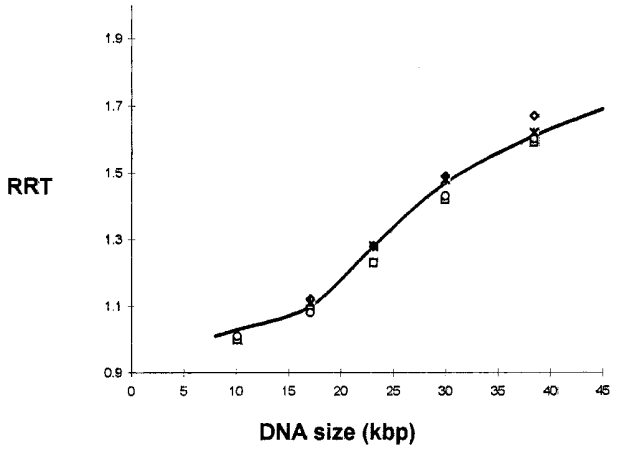

Figure 2. RRT (relative retardation time) values calculated using eqs 10 and 12 in relation to the DNA molecule size (kb) for a packing particle size fixed at 9 μm and a flow rate equal to 0.3 mL/min (—). Experimental data reported by Hirabayashi et al.[16] using four different porous polymer packings (exclusion size: 3×10^3 (*), 4×10^4 (□, ◇), and 3×10^5 Da (○)) having the same particle diameter, 9 μm.

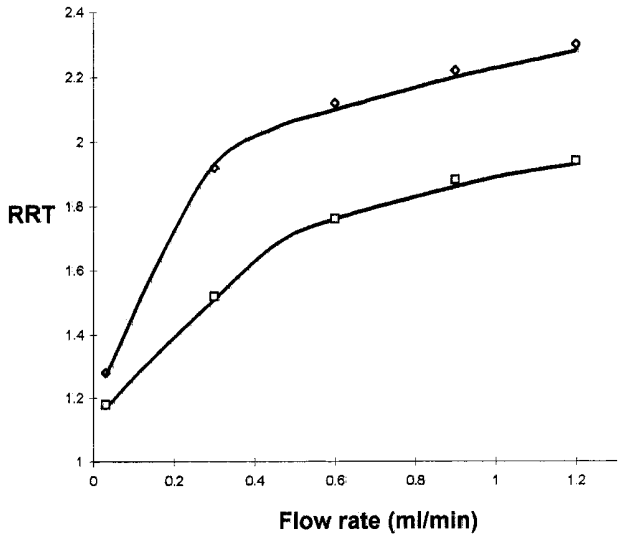

Figure 3. Theoretical flow rate dependence on RRT for two DNA fragments 17.05 and 29.95 kb for a particle diameter of 4 μm (—). Experimental data obtained using a Zorbax column (see Experimental Section) with a particle diameter equal to 4 μm for the DNA fragments 17.05 (□) and 29.95 kb (◇).

of the columns were used to estimate the RRT values with the measured values for the various DNA fragments at the different flow rates. 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 average θ angle can be calculated from the directional change factors. For example, the θ value obtained for a packing particle size fixed at 9 μm was equal to around 95°.

Size Dependence on RRT. The average theoretical and observed RRT values reported previously[16] were plotted against the DNA fragment size for a fixed packing particle size and a flow rate. For example, Figure 2 shows the curve determining the size dependence on RRT for a flow rate equal to 0.3 mL/min. As described by the model, the RRT values increased for a constant particle diameter and flow rate when the size of the DNA chain increased. The zones of the sigmoidal-like curve corresponded to the two cases reported in the theoretical section for the polymer progression into the column, i.e., when $\Gamma_c > \Gamma_o$ (case 1) and $\Gamma_c < \Gamma_o$ (case 2). The critical value of the steady-state extension R_c when the transition between case 1 and case 2 occurred for $\Gamma_o = \Gamma_c$ was determined by

$$R_c = d_p K'' \ln(1/K'K'') \qquad (15)$$

R_c was only dependent on the particle arrangement of the used columns. At a flow rate equal to 0.3 mL/min, this R_c value corresponded to a critical value of DNA with a size equal to around 20 kb (DNA_c).

Above the transition point, the variation in RRT with the DNA size followed eq 10. When the DNA size was small in such a way that $R \ll d_p$, RRT → 1. This was the situation for a macromolecule with a size of <10kb. When the size increased from 10 kb to DNA_c, RRT strongly increased with the DNA length. This was brought about by two phenomena: (i) a functional dependence of RRT on R, when $R \rightarrow R_c$ according to eq 10, and (ii) for a constant flow rate, a greater increase in the steady-state extension R for large DNA rather than small DNA molecules as defined by eq 13. This second mechanism associated with the DNA stretching enhanced the magnitude of the slope predicted by the eq 10 function for the R value.

Below the transition point, a weaker increase in the RRT values was observed in relation to the DNA length. This was in part consistent with the shape of the eq 12 function. However, the additional mechanism described for case 1, i.e., great elongation dependence for the large molecules on the hydrodynamic flow, provided a steeper slope than that predicted by eq 12, explaining the linear portion in the final zone of the curve.

Flow Rate Dependence on RRT. The liquid velocity dependence on the relative retardation time was shown by plotting the predictions of the theory and our experimental data in relation to the flow rate for a 4 μm particle size and two DNA chain sizes (Figure 3). The curves reproduced by the calculation corresponded to the stretch-dependent retardation of the DNA strands when the liquid velocity increased. At a low flow rate, the DNA chain was in a random coil configuration and then RRT → 1. As the flow rate increased, the molecule was stretched and RRT was related to the steady-state extension as defined by eq 13. Above R_c corresponding to a flow rate of <0.3 mL/min for the two fragments, the RRT variation was governed by the eq 10 function with a steep slope and eq 12 was implied in the DNA retardation below R_c (flow rate >0.3 mL/min). As shown in Figure 3, it is important to note that, in this chromatographic procedure, the flow rate increase enhanced or maintained the separation efficiency with an associated decrease in the analysis time (decrease in t_{NR}). This constitutes the advantage of the hydrodynamic principle over the equilibrium principle, which is the basis of the classical chromatographic separation.

The use of the concept derived from the reorientation time of biopolymers makes it possible to obtain useful information on this new hydrodynamic process. To the best of our knowledge, this model constitutes the first case of analysis of DNA in a highly

dense medium dependent on a hydrodynamic flow. The hydrodynamic mechanism of DNA separation implied in the slalom chromatography seems to be close to the mechanism of nucleic acid molecular sieving by high-performance capillary gel electrophoresis using Pluronic liquid crystals as replaceable media.[22,23] Some common features are retrieved such as the DNA travel through the interstitial spaces created by a compact arrangement of spherical obstacles. Moreover, this electrophoretic procedure is able to extend the analysis possibilities to smaller DNA molecules than in slalom chromatography due to the smaller diameter of the spherical obstacles (18 nm). Thus, our theoretical model could be applied to this recent electrophoretic technique or capillary electrochromatography (which combines both the chromatographic and electrophoretic principles) to enhance the opportunities of DNA separation in a hard and dense structure. As well, the heterogeneous nature of the packing in a classical chromatographic column limited the accuracy of our model due to the use of approximations for the DNA progression through the interstices. A more refined analysis of the hydrodynamic phenomenon could be attained using highly miniaturized analytical systems. For example, the nanocolumns for liquid chromatography microfabricated by lithography/etching technology[24] generate a very precise and clearly defined architecture of particles and channels. This type of microchip could then provide valuable information on the values of r, θ, and directional change factors as well as a more complete model for hydrodynamic separation.

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