Study of the steric partition coefficient in size-exclusion chromatography by Monte Carlo simulation

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Monte Carlo generation of polymer chains allows evaluation of the steric partition coefficient, K, for any pore shape and any chain flexibility. This in turn enables the chromatographic radius of macromolecules, R_c, to be defined as the radius of a sphere with the same K value and allows the ratio R_c/R_η to be studied. R_η is defined as the radius of the sphere with the same product $[\eta]M$, where M is the molar mass and $[\eta]$ the intrinsic viscosity. R_c is shown to depend on the pore geometry. For a given pore geometry, R_c/R_η is not strictly independent of the flexibility and the relative thickness of the macromolecule. Experimentally, for flexible polymers, the so-called universal calibration is often found to work well. However, simulations show that the universal calibration is not strictly valid and deviations are expected in particular when the global flexibility of standard and sample are different.

(Keywords: size-exclusion chromatography; universal calibration; steric partition coefficient)

INTRODUCTION

Size-exclusion chromatography (s.e.c.) is a powerful tool for characterizing the molar mass distribution of polymers. Recent technological improvements have made this technique even more attractive. Unfortunately this method relies on a calibration based on monodisperse standards and does not yield the absolute molar masses when the studied macromolecule is different from that used in the calibration. Universal calibration (UC), first proposed by Benoît *et al.*[1], apparently solved the problem by simply relating each macromolecule size (i.e. each elution volume) to a product $[\eta]M$. Although the on-line viscosity measurements[2–4] raise experimental problems[5,6], especially with high performance column sets, intrinsic viscosity ($[\eta]$) measurements can nowadays be reliably performed[7,8].

It has been claimed by many authors (first by Grubizic *et al.*[9]) that UC is valid experimentally whatever the chemical nature and the structure of the polymer[10]. However, from a theoretical point of view the situation is different. Casassa and Tagami[11] and Giddings *et al.*[12] assume that the elution process is governed by the equilibrium distribution of the solute between the mobile phase and the stagnant phase inside pores. The equilibrium is characterized by the partition coefficient, K, defined as the ratio between the concentration inside the pores and the concentration outside the pores. Using statistical mechanics, they have calculated K for random flight linear and branched chains as well as for rods, confined in pores with simple geometries. Casassa showed that UC is valid for any linear or branched macromolecule containing a large number of statistical segments. The results of Giddings *et al.*[12] lead to different partition coefficients for a thin rod and for a flexible chain with the same viscometric radius. These idealized theoretical calculations indicate that UC can be applied to polymers of different architecture, but that rigidity might be a limiting factor.

The aim of this paper is to study the influence of the flexibility of linear chains on the chromatographic radius and its relation to the viscometric radius through a Monte Carlo simulation of the size-exclusion phenomenon.

DEFINITION OF DIFFERENT SIZES OF A MACROMOLECULE

A single macromolecule has a complex temporal and spatial distribution of conformations and only average dimensions can be calculated or measured. A special mention is given to the radius of gyration, which corresponds to a clear geometric definition. The simplest way to compare sizes, as measured by different experimental techniques, is to define the corresponding size as the radius of a sphere with uniform density which has the same measured property as the macromolecule. The following sizes can be defined.

1. The *Stokes radius*, R_s, obtained from measurements of the translational diffusion coefficient:

$$R_s = \frac{kT}{6\pi\eta_0 D_t} \tag{1}$$

where k, T, η_0 and D_t are the Boltzmann constant, the absolute temperature, the solvent viscosity and the translational diffusion coefficient, respectively.

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2. The *viscometric radius*, R_η, obtained from mass and intrinsic viscosity measurements:

$$R_\eta = \left[\frac{3[\eta]M}{10\pi N_a}\right]^{1/3} \quad (2)$$

where N_a is the Avogadro number.

3. The *chromatographic radius*, R_c, obtained fron s.e.c. (R_c is the radius of the sphere that would have the same elution volume for a given pore geometry).

Using these definitions, UC applies if the relation between R_η and R_c does not depend on the nature of the sample (linear or branched, flexible or rigid). For linear chains, a good estimate of R_η can be obtained from the theoretical work of Yamakawa and Fujii[13]. R_c values have been calculated using a computer simulation in a wide range of realistic situations, especially for chains of variable stiffness ranging from freely rotating segments to rigid rods.

PRINCIPLE OF SIMULATION

The key parameter for s.e.c. is the partition coefficient K related to the elution volume: $V_e = V_0 + KV_p$, where V_e is the solute elution volume, V_0 the void volume and V_p the pore volume of the column. For rigid spheres, K is simply the volume fraction of the pores accessible to the centre of mass. For any other shape, K is best defined as the equilibrium constant for solute exchange from intrapore volume to bulk solvent as proposed by Casassa:

$$\text{Solute in bulk solvent} \rightleftharpoons \text{Solute inside pore}$$
$$K = \frac{[S]_p}{[S]_b}$$

K has been calculated as follows. For a given pore geometry, a macromolecule is created by randomly choosing a starting point inside the pore volume. A random walk modified to account for conformational constraints is generated from this point. A macromolecule can be considered inside the pore if all segments remain inside the pore. Therefore K appears as the value of the fraction of successful trials, for a large number of trials. R_c is the radius of a sphere that would have the same elution volume as the macromolecule and whose centre of mass therefore has access to the same fraction K of the pore volume. For a number of pore geometries, there is a simple relation between R_c and K:

(i) for a cylindrical pore of circular section with radius R_p or of square section with side $2R_p$:

$$K = (1 - R_c/R_p)^2 \quad (3)$$

(ii) for a spherical pore with radius R_p or a cubic pore with side $2R_p$:

$$K = (1 - R_c/R_p)^3 \quad (4)$$

Different chain conformations can be constructed. Two limiting conformations are fully rigid rods that contain only one segment and ideal chains (random walks). For more realistic chains the orientation of the segment $i+1$ is defined by two angles (α and β). α is the angle between segments i and $i+1$ and β is the angle between the plane defined by segments $i-1$ and i, and the plane defined by segments i and $i+1$. Fixed values of α and β give a regular helix if β is not equal to 0 or π. Keeping α fixed and varying β randomly between 0 and 2π gives chains with freely rotating segments. Low values of α generate worm-like chains. Excluded volume effects are not taken into account during chain construction.

The persistence length is the classical parameter used in order to characterize local chain rigidity. Here, we define a parameter P reflecting the situation for the whole chain as: P = contour length/persistence length. For chains containing N freely rotating segments one obtains:

$$P = \frac{2N(1-\cos\alpha)}{(1+\cos\alpha)}$$

P is equal to zero for a fully rigid rod and to infinity for a random coil (N infinite).

The principle of the simulation is as follows: after choosing the values of N and α, chains are generated inside a pore of fixed geometry and size until a stable value of K is obtained. By using different pore sizes, K values can be varied between realistic limits ($0.1 < K < 0.9$). The chromatographic radius can be calculated using relations (3) and (4) given above. The radius of gyration is also calculated from the chain construction so that R_c/R_g values can be obtained.

COMPARISON WITH THEORETICAL RESULTS

The validity of the simulation procedure can be tested by comparison with explicit analytical results, available in limiting cases. As the position of each segment is explicitly known, the radius of gyration of each molecule can be calculated by direct summation. By choosing a pore of infinite size there is no constraint on the population and our results can be compared with theoretical values[14]. For a chain with N freely jointed segments with length l, R_g is given by:

$$\langle R_g^2 \rangle = \frac{N(N+2)l^2}{6(N+1)}$$

Small values of N allow accurate checking of the uniformity of orientation randomness. After generation of 5×10^4 chains, deviation with theory was less than 0.1%. For a chain with a large number of freely rotating segments:

$$\langle R_g^2 \rangle = \frac{N(N+2)l^2}{6(N+1)} \frac{1+\cos\alpha}{1-\cos\alpha}$$

Using values of N from 100 to 500 and α from 0.2 to $\pi/2$, deviation with theory is less than 0.1% after generation of more than 10^5 chains. Pure helices are indeed obtained when β is fixed.

Concerning theoretical values of the partition coefficient in simple pores, two special cases have been investigated in the literature. For random coils containing large numbers of segments in cylindrical pores, Casassa predicted[15]:

$$K = 4 \sum_{i=1}^{\infty} \frac{1}{\alpha_i^2} e^{\frac{-\alpha_i^2 N l^2}{6R_p^2}}$$

Here α_i is a root of $J_0(\beta) = 0$ and J_0 is a Bessel function of the first kind and order 0. In the case of rigid rods in cylindrical pores Giddings *et al.*[12] derived expressions for K which are combinations of elliptical integrals depending only on $1/R_p$. Taking fixed values of the segment length ($l = 1$ in both cases), the molecular size to pore size ratio was varied for a one-segment rod and

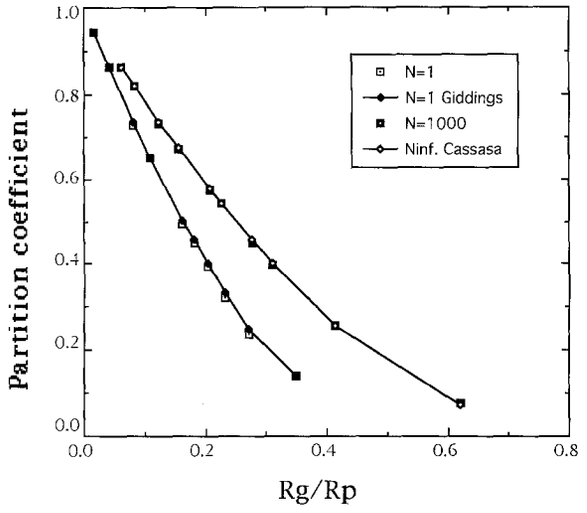

Figure 1 Partition coefficient K as a function of the ratio of the radius of gyration to the pore radius (R_g/R_p) for two values of the segment number N. The results of Casassa and Tagami[11] and Giddings *et al.*[12] are included

a 1000-segment chain. *Figure 1* shows that there is close agreement between simulation and theory in these two limiting cases.

The limiting value $K=1$ is reached as the pore becomes infinitely large compared with the chain. Geometrical details have no influence and the problem is reduced to the estimation of the depletion layer. For a polymer solution near a non-interacting wall there is a zone where the segment concentration $C(x)$ increases from 0 for $x=0$ to C_{bulk} for $x=\infty$. The depletion layer D is defined by:

$$D=\frac{1}{C_{bulk}}\int_0^\infty C(x).dx$$

For a population of rigid spheres the depletion layer is simply the radius of the sphere and therefore when $K\to 1$, $R_c\to D$. For any kind of macromolecule, Casassa[16] established that $D=X/2$, X being the mean projection of the unconfined molecule on an axis. For a thin rigid rod one obtains $D=L/4$ and $R_c/R_g=0.86$. For a Gaussian chain:

$$D=\frac{1}{2}\left(\frac{8Nl^2}{3\pi}\right)^{1/2}$$

and $R_c/R_g=1.13$. Simulation of these two situations with $K=0.98$ recovers these values within 0.5%.

In this study, the size of the test population is always chosen such that the error is less than 1%. It depends mainly on the number of segments, the angle between segments, and the value of the partition coefficient. *Figure 2* illustrates a representative case for a chain with 100 segments of length 1 and with $\alpha=\pi/2$, in a cylindrical pore of radius 15. A good balance between time of simulation (which is roughly proportional to N) and accuracy can be obtained with 5×10^4 chains.

RESULTS AND DISCUSSION

It is now possible to study, using our simulation procedure, situations that cannot be easily handled analytically, i.e. the influence of pore geometry and chain flexibility.

Influence of pore geometry

Observations by electron microscopy have been performed on various column packings: controlled porous glass[17-20], methacrylate gels[21], hydrophilic polyether gels[22] and styrene–divinylbenzene gels[23]. These packings are found to consist of beads obtained by partial fusion of small irregular particles. This produces very deep, tortuous channels with irregular cross-sections but never with very thin protuberances or sharp angles. To take into account the geometry encountered along a given pore, a reasonable model would be a combination of long cylinders with circular or square sections and with more compact closed volumes (spheres or cubes). These four pore geometrical models were tested in order to establish whether an absolute size independent of the pore geometry can be estimated from s.e.c. in realistic situations ($0.1<K<0.9$).

Two kinds of molecules have been tested: ideal random coils and rigid rods. In order to evaluate the chromatographic size R_c for molecules of different absolute size and geometry, we have chosen to compare

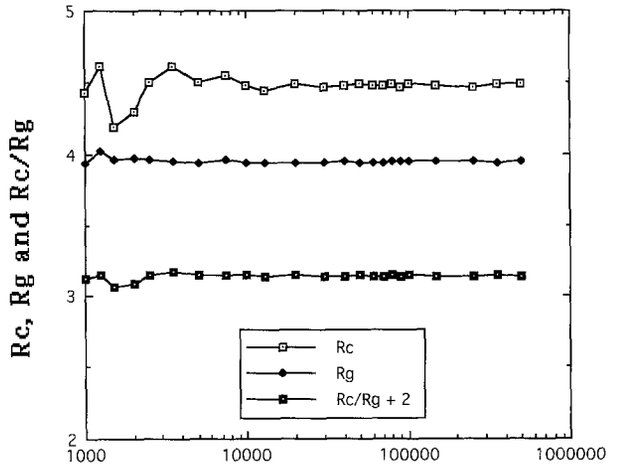

Figure 2 Chromatographic radius R_c, radius of gyration R_g and ratio R_c/R_g as a function of the number of generated chains (simulations with 100 segments of length $l=1$, and angle $\alpha=\pi/2$ in a cylindrical pore of radius $R_p=15$)

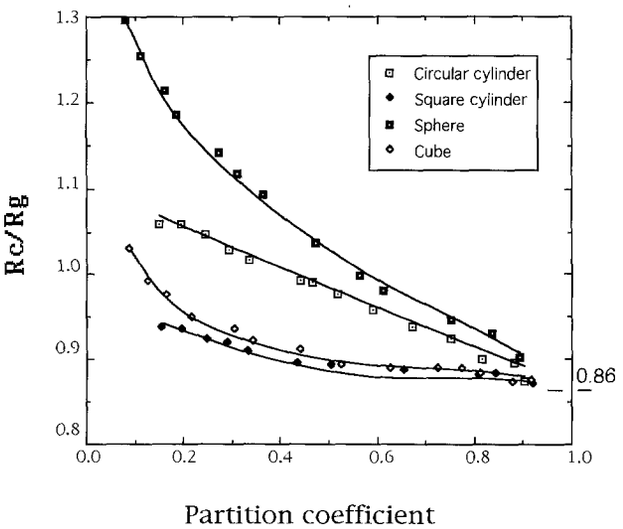

Figure 3 Ratio of the chromatographic radius to the radius of gyration (R_c/R_g) as a function of the partition coefficient for rods in pores of various geometries. Curves are obtained with length of rod $l=1$ and various pore sizes, R_p. The limiting value for $K=1$ is indicated

the values of the dimensionless parameter R_c/R_g in different practical situations. To allow the partition coefficient to vary between 0.1 and 0.9, for rods with length taken as unity (*Figure 3*), the size of the pore has been varied from 0.5 to 5 for cylindrical pores with circular section, from 0.9 to 12 for cylindrical pores with square section or cubic pores, and from 0.5 to 7 for spherical pores. For chains with 100 freely joined segments of length 1 (*Figure 4*), R_p has been varied from 7 to 80 for cylindrical pores with circular section, from 12 to 140 for cylindrical pores with square section or cubic pores, and from 7.7 to 90 for spherical pores. For flexible chains, R_c/R_g varies between 1 and 1.2. For rigid rods, R_c/R_g shows a larger variation between 0.86 and 1.3. Additionally, for each pore geometry R_c/R_g depends weakly on K. These results imply that s.e.c. does not give absolute size, but R_c values are also influenced by the shape–shape interactions between porous matrix and molecules.

The fluctuations of R_c/R_g as a function of the pore geometry can be explained as follows for rods: a rod in a spherical or cubic pore can only take conformations with its centre of mass near the centre of the pore. In a cylindrical pore with circular section, rods can be oriented along the axis of the pore. So K is greater in that case for the same pore section and R_c/R_g is smaller for a given K. In a cubic or square-section pore, rods can be oriented along diagonals. Therefore the same phenomenon is observed for smaller values of K. For chains with 100 segments the situation is a little different: they cannot be easily oriented along a given axis because only a small fraction of chains is stretched. Therefore differences between pore geometries are qualitatively the same as for rods, but to a smaller extent.

Influence of flexibility on R_c/R_g

As it appears that the ratio R_c/R_g is different for coils and rigid rods, it is interesting to study more precisely the intermediate situations. The influence of the overall chain flexibility parameter on R_c/R_g is seen in *Figure 5*, for given values of the partition coefficient and a given

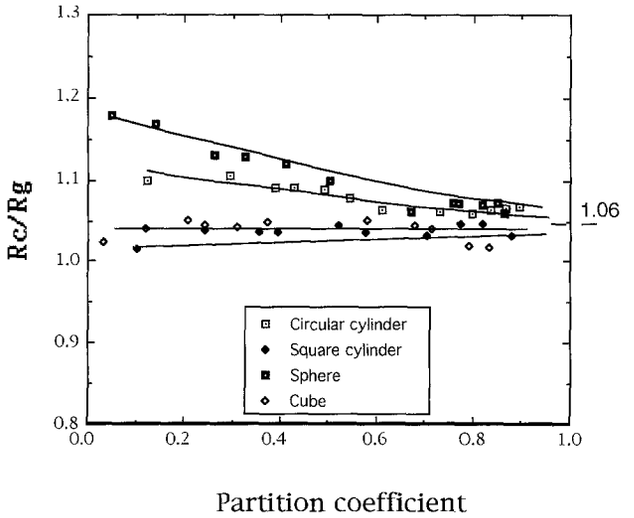

Figure 4 Ratio of the chromatographic radius to the radius of gyration (R_c/R_g) as a function of the partition coefficient for chains with $N=100$ segments in pores of various geometries. Curves are obtained with length of segment $l=1$ and various pore sizes, R_p. The limiting value for $K=1$ is indicated

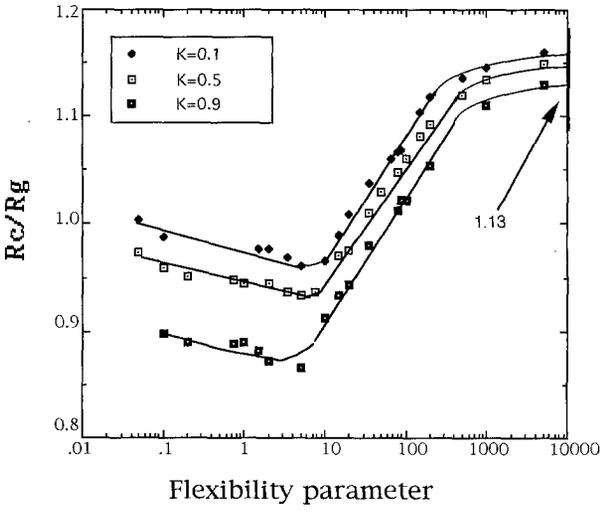

Figure 5 Ratio of the chromatographic radius to the radius of gyration (R_c/R_g) as a function of the flexibility parameter P for a cylindrical pore geometry. Results are plotted for three values of K

pore geometry. P can be varied by either changing the angle α or the number of segments N. It has been checked that R_c/R_g depends only on P. For P below 200, N was fixed at 100 and α was varied. For higher values of P a fixed value $\alpha=\pi/2$ was used and N was varied. The shape of the curves does not depend very much on the value chosen for K and similar curves have been obtained for other pore geometries. For small values of P (i.e. for rigid molecules) R_c/R_g does not depend much on P. A transition is observed around $P=10$. Beyond this value a rapid increase of R_c/R_g is obtained and the curves seem to level off for very high values of P. In particular, for $K=0.9$ and $P=5\times10^3$, R_c/R_g values are very close to the theoretical limit given by depletion layer calculations ($R_c/R_g=1.13$).

Influence of flexibility on R_c/R_η

In order to calculate R_η using equation (2) we have used the numerical expressions proposed by Yamakawa and Fujii[13]. They have expressed intrinsic viscosity for worm-like chains as a function of contour length (L), mass per unit length (M_l), and diameter (d). To take into account in the simulations a finite value of the chain diameter d, it is sufficient to consider that at the pore wall the chains are excluded over a distance $e=d/2$. Assuming that R_c is the chromatographic radius of a thin molecule in a pore of radius R_p, then for the corresponding thick molecule the chromatographic radius will be R_c+e in a pore of radius R_p+e. This simple transformation allows us to compare R_c obtained by simulation with R_η for chains with finite thickness. For a thin rigid rod of length L, effects of finite diameter vanish rapidly. For $L/d>20$, R_c/L does not depend on L and is fixed for a given pore geometry and K value. The limiting expression of R_η for large values of L/d is[24]:

$$R_\eta^3 \approx [\eta]M \approx \frac{L^3}{\ln(L/d)}$$

Therefore R_η/L depends on L/d even for thin rods and theoretically the ratio R_c/R_η increases for rigid rods of constant diameter and increasing length:

$$\frac{R_c}{R_\eta} \approx \left(\ln\frac{L}{d}\right)^{1/3}$$

More generally we can study the situation for any worm-like chain with constant local properties (diameter d and persistence length q) and various contour lengths (L), i.e. various flexibility $P = L/q$. *Figure 6a* represents the case of an arbitrary very thin molecule ($q = 100$, $M_l = 20$, $d = 0.15$) for which P varies from 0.05, i.e. $L/d = 33$ (characteristic of a rod), to 1000 (characteristic of a coil). As R_c is slightly dependent on K, curves corresponding to three values of K are plotted. Up to $P = 1$ ($L/d = 660$) the variation in R_c/R_η is similar to that of a thin rigid rod, which is not surprising, as R_c is valid for a rod up to $P = 5$ (*Figure 5*). For higher P values, the viscometric behaviour becomes progressively that of a coil and there is partial compensation between the variations of R_η and that of R_c. For $K = 0.9$, values obtained for larger values of P are compatible with the limiting value derived from a calculation of the depletion layer. In effect, in this case $R_c/R_g = 1.13$ and for Gaussian coils $R_\eta/R_g = 0.78$, therefore $R_c/R_\eta = 1.45$.

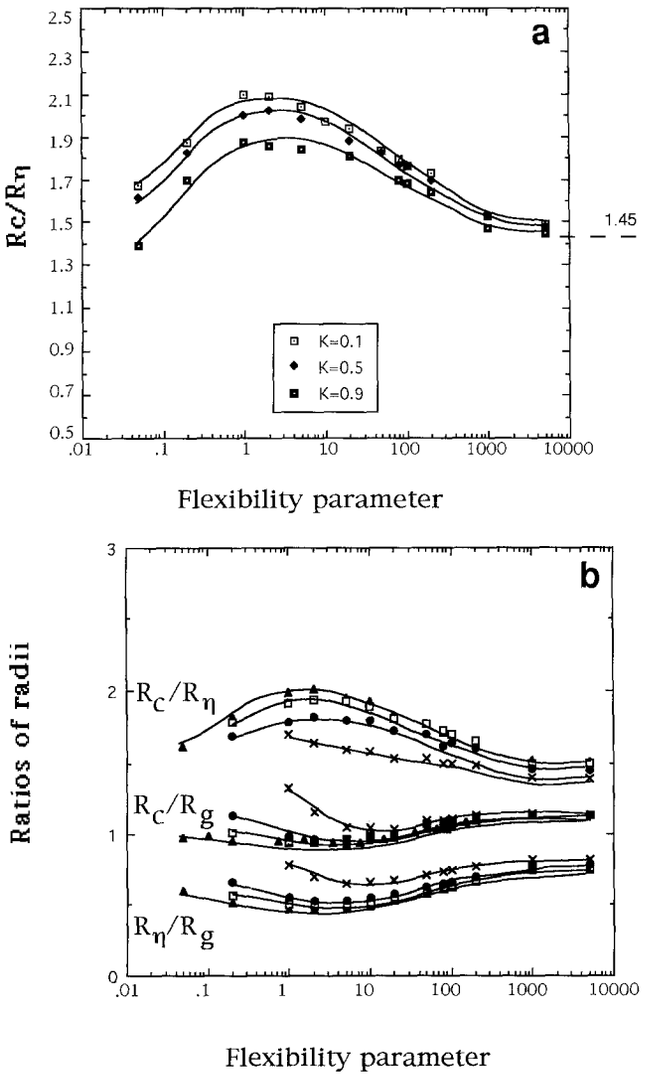

Figure 6 (a) Ratio of the chromatographic radius to the viscometric radius (R_c/R_η) as a function of the flexibility parameter P. Curves are obtained by combination of R_c/R_g derived from simulations and R_η/R_g from the theoretical work of Yamakawa and Fujii[13], using mass per unit length $M_l = 20$, diameter $d = 0.15$ and persistence length $q = 100$. Results are plotted for three values of K. (b) Ratios of the chromatographic radius to the viscometric radius (R_c/R_η), the viscometric radius to the radius of gyration (R_η/R_g) and the chromatographic radius to the radius of gyration (R_c/R_g) as a function of the flexibility parameter P. Curves are obtained by combination of R_c/R_g derived from simulations and R_η/R_g from the theoretical work of Yamakawa and Fujii[13], using mass per unit length $M_l = 20$, persistence length $q = 100$ and four values of diameter d: ▲, 0.15; □, 0.33; ●, 1; ×, 10. Results are plotted for partition coefficient $K = 0.5$

R_η/R_g, R_c/R_g and R_c/R_η are plotted in *Figure 6b* as a function of the flexibility parameter P. R_c/R_g has been derived from simulations, and R_η/R_g from the theoretical work of Yamakawa and Fujii[13], using $M_l = 20$, $q = 100$ and four values of d (0.15, 0.33, 1 and 10). The partition coefficient K was 0.5. The smaller the value of q/d, the smaller the fluctuations of R_c/R_η. Yamakawa and Fujii's calculations for a worm-like chain are only valid for $q/d > 10$ and therefore do not describe the situation of flexible polymers for which the persistence length is in the same order of magnitude as d. However, by extrapolation, these results clearly suggest that the ratio R_c/R_η stabilizes for lower values of q/d. This is merely due to compensation between variations in R_c/R_g and R_η/R_g.

For realistic chains, such as DNA ($q = 550$, $M_l = 195$, $d = 23$), R_c/R_η has been estimated from $P = 0.2$ to 100 (corresponding to molecular weights from 2×10^4 to 1×10^7). In this range, the configuration of DNA changes from a rod to a statistical chain (*Figure 7*). This macromolecule has been studied extensively and UC does not seem to be valid in practice[25]. The differences between the 'rod' behaviour and the 'chain' behaviour are less important here, but still exist: R_c/R_η increases by a factor of ~ 1.2 when P decreases from 100 to 0.2.

Consequences for universal calibration

UC entails that two macromolecules that have the same elution volume (same R_c) for a given column (with a fixed pore geometry), have the same R_η. For linear, highly flexible, high molar mass polymers, UC is experimentally well established. From a theoretical point of view this corresponds to the situation where all the radii, R_η, R_g and R_c, have reached their asymptotic behaviour and R_c/R_η no longer depends on the details of the polymer structure. In the simulations, we have observed this behaviour for P values higher than 1000.

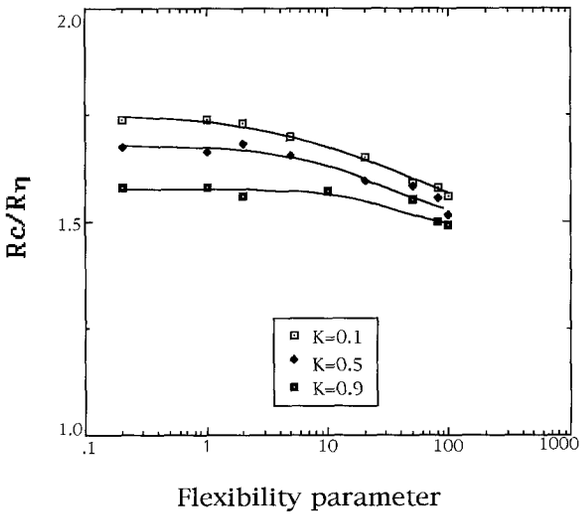

Figure 7 Ratio of the chromatographic radius to the viscometric radius (R_c/R_η) as a function of the flexibility parameter P. Curves are obtained by combination of R_c/R_g derived from simulations and R_η/R_g from the theoretical work of Yamakawa and Fujii[13], using chain parameters of DNA (mass per unit length $M_l = 195$, persistence length $q = 550$ and diameter $d = 24$). Results are plotted for three values of K

Medium or low molar mass flexible polymers have q/d values near 1 and P values between 10 and 1000. As pointed out previously, extrapolation of the results suggests that R_c/R_η tends to be stable in this P range. However, this can in no way be considered as proof for the validity of UC. Moreover, recent experiments and theoretical work on the basis of the helical worm-like chain model[26,27] indicate that R_η/R_g varies specifically from one polymer to the other and so it would be surprising to always have exact compensation between variations in R_c/R_g and R_η/R_g. Further studies in this area are in progress.

The situation is clearer when the studied polymer has a very different flexibility from the standard. In that case, for the same R_c value R_c/R_η is different for a semirigid polymer of low flexibility parameter and for a flexible standard, and therefore UC is not valid. For example, on a given arbitrary column we can elute at the same elution volume, i.e. same R_c, either rigid DNA (molecular weight, $MW=27\,000$, $P=0.5$ and $R_c/R_\eta=1.68$) or highly flexible polyoxyethylene ($MW=50\,000$, $P>300$ and $R_c/R_\eta=1.45$). If UC is applied, the calculated value of R_η for DNA is 15% too high and the calculated mass, which is proportional to R_η^3, is overestimated by 50%. However, for very high MW, the differences vanish progressively as the flexibility parameter of DNA increases.

For polymers resembling spheres with uniform density, such as globular proteins, the situation is worse as R_c/R_η is formally 1 for a sphere and near 1.5 for a flexible standard. In this case the error made by applying UC will become very important. Experimentally, however, this is obscured by electrostatic effects that strongly modify the accessible pore volume depending on the ionic strength.

SUMMARY

In the case of a purely steric exclusion mechanism, Monte Carlo generation of freely rotating chains allows evaluation of the partition coefficient for any pore shape. In this way the chromatographic radius of a macromolecule can be defined as the radius of the sphere having the same K value in the same pore. In most realistic situations, the results show that R_c is not very different from R_g, but noticeable variations occur depending on K and on the exact pore geometry. Keeping the latter parameters fixed, we have studied the influence of the flexibility of the chain on R_c/R_g. Differences of up to $\sim 25\%$ are observed between the values for a rigid rod and a flexible coil. Results for infinitely thin chains can be adjusted so as to be applicable to chains with finite diameter. R_c can therefore be compared with R_η, obtained from theoretical calculations. The evolution of R_c/R_η has been presented as a function of the flexibility. For realistic semirigid polymers, the variation in R_c/R_η is moderate, mainly due to the fact that low MW macromolecules are relatively thick rods.

Nevertheless, the values of R_c/R_η depend significantly on the architecture of the analysed polymer (semirigid, coil or globular), and it is likely that recent developments in s.e.c. multidetection, associating an absolute mass detector, a viscometric detector and a concentration detector, will contribute to progress in polymer shape determination.

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