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Nonionic polysaccharides as calibration standards for aqueous size exclusion chromatography

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Size exclusion chromatography may be used to determine molecular size or mass of solutes. The validity of the method depends on the correct choice of macromolecular standards used to calibrate the chromatographic column. This calibration is an experimental determination of the relationship between the molecular dimensions and the peak migration velocity of the solute, in practice often presented as a semi-logarithmic plot of log(MW) vs elution volume, but more fundamentally expressed as the dependence of for example, the Stokes radius (R_S), or the viscosity radius (R_η) on the chromatographic partition coefficient, K_{SEC}. The validity of this calibration rests on the absence of enthalpic interactions between the standards and the stationary phase and the ability to determine the standards' molecular dimensions and/or mass in a nonambiguous way. Nonionic polysaccharides are ideal for this purpose, and furthermore provide an excellent paradigm for studying the role of molecular architecture in the relationship between K_{SEC} and R_η or R_S.

INTRODUCTION

Size exclusion chromatography (SEC) is both a liquid chromatographic separation technique and at the same time, a molecular weight method. There are, however, many *caveats* associated with the second application. The two most serious ones are (a) that the relationship between solute size and peak migration velocity not be perturbed by interactions between the solute and the stationary phase; and (b) that the relationship between solute size and mass can be exactly determined. In one of the most popular uses of aqueous SEC—protein characterization—both of these factors are problematic. Since all aqueous SEC stationary phases bear some surface charge, typically negative, both repulsive and attractive electrostatic forces between the protein and the packing can affect the retention time (Dubin, 1988). In addition, hydrophobic interactions can lead to enhanced retention for certain proteins on polymer-based packings. With regard to the second factor, (b), the relationship between the size and mass of proteins is obscured by the irregular geometry of globular proteins, such that many of them can be modeled, hydrodynamically, as ellipsoids with axial ratios between two and three (Cantor & Schimmel, 1980), whereas others may approach more nearly spherical symmetry.

For the foregoing reasons, the calibration of SEC columns with globular protein standards, while obviously appealing if the analytes of interest are globular proteins, is not entirely satisfactory. Published calibration curves (e.g. log MW vs retention volume) typically show considerable scatter (the severity of which is somewhat cosmeticized by the customary use of semi-logarithmic calibration curves). A least squares fit curve is fundamentally incorrect, because one cannot be confident that the data more heavily weighted in this process are indeed the ones that correspond to more 'ideal' behavior. For example, if variable protein adsorption was the main source of deviations from the hypothetically correct calibration curve, then the points to the right hand side of the curve should be excluded from the averaging process. To some extent, the deviations among the data may also reflect variations in geometry for the proteins. Resolution of this last effect from the effects of adsorption or repulsion is a challenging task.

The ideal standards for SEC should possess the following properties:

(a) Enthalpic interactions with the stationary phase should be negligible.
(b) The solutes should be spherical, and monodisperse with respect to size.
(c) The solutes should be well characterized with respect to size.
(d) Changes in effective solute size due to aggregation or scission should be unimportant.

Solutes that meet the requirements listed above could be used to establish an 'ideal SEC' curve for any

column. The retention time for any solute could then be related with confidence to its size. In the case of solutes characterized by other techniques, their deviations from the 'ideal curve' could be confidently ascribed to either interactions with the stationary phase or departure from spherical symmetry.

The foregoing requirements are best met by nonionic polysaccharides. In the abundant SEC literature, there is no report of adsorption or anomalous retention of e.g. dextran on any sort of SEC packing. Nonionic polysaccharides normally do not aggregate strongly and are relatively stable with respect to shear degradation. Thus, nonionic flexible chain polysaccharides, namely, dextran, have been used for calibration of SEC columns almost from the time of inception of the technique. However, the virtues of polysaccharides that do not have Gaussian chain statistics as solutes for testing SEC theory are less well recognized. While other biopolymers may possess conformations that hydrodynamically approximate rods, spheres or ellipsoids, they also possess electrostatic and/or hydrophobic character, which complicates the interpretation of their chromatographic properties. Ficoll and schizophyllan, for example, are among the few water-soluble uncharged, nonhydrophobic macromolecules with well-defined geometry. The hydrodynamic properties of Ficoll suggest its resemblance to a non-compressible sphere (Holter & Møller, 1956) which makes its retention behavior an excellent test of SEC theory. Uncharged rodlike helical polysaccharides provide a paradigm for the SEC of asymmetric solutes, since, unlike DNA, they migrate in an SEC column without the influence of electrostatic effects.

Neutral polysaccharides have been used as probes of permeation for some time in areas of investigation outside of SEC. Ficoll, a highly branched copolymer of sucrose and epichlorohydrin (Laurent & Granath, 1967; Laurent, 1967) has been the subject of numerous studies. Deen and co-workers measured the effective diffusion coefficients of dextran and Ficoll through track-etched membranes (Deen *et al.*, 1981; Bohrer *et al.*, 1984; Davidson & Deen, 1988). On the basis of such work it has been concluded that Ficoll behaves approximately as a rigid sphere, in contrast to the behavior of dextran.

While the objective of the foregoing studies was an understanding of the fundamental aspects of hindered diffusion in model systems, similar experiments have been carried out in complex biological systems, or under physiological conditions. Diffusion coefficients in cytoplasm have been measured with dextran (Luby-Phelps *et al.*, 1986) and Ficoll (Luby-Phelps *et al.*, 1987). The permeability of capillaries and membranes has been studied using fractionated dextrans (Luby-Phelps *et al.*, 1987). The permselectivity of the glomerular capillary wall has been studied extensively by Robertson and coworkers, using neutral dextran as well as its ionic derivatives (Chang *et al.*, 1986; Bohrer *et al.*, 1979). Dextran MW fractions were also used to measure the permeability of human arterial endothelial cell monolayers (Langeler & Van Hinsbergh, 1988). The holes in human erythrocyte membrane ghosts were studied by measuring the rate of efflux of dextrans from Hb-free ghosts, leading to characterization of the size and geometry of the holes (Lieber & Steck, 1982). Characterized MW fractions of nonionic polysaccharides have been frequently used to study the permeability under flow of synthetic polymer materials. The molecular weight cut-off and flux properties of polysulfone ultrafiltration membranes have been measured using dextran standards (Nobrega *et al.*, 1989). A similar technique, combined with SEC, was applied to hollow-fiber ultrafiltration membranes (Ohya *et al.*, 1991).

The characterization of porous media can be carried out by equilibrium methods. The dependence of the degree of permeation on MW contains information about the pore size distribution. This technique, sometimes called 'inverse SEC', has been applied to networks of insoluble collagen from human skin (Pearce & Laurent, 1977); and to water-swollen cellulosic materials (Stone *et al.*, 1969) using dextrans. As Kuga has pointed out (Kuga, 1988), a number of inverse SEC experimental techniques can be employed, some completely static, and others resembling chromatography. Perhaps the most convenient is a 'mixed solute exclusion' method (Kuga, 1988), in which the porous material is equilibrated with a mixture of oligo- and polysaccharides, and SEC is used to characterize the MW distribution of the probe mixture before and after equilibration.

The above-mentioned reports are among the very few that relate in any way the chromatographic behavior of polysaccharide MW standards to their behavior as probes of the flux through porous media. The relationship between these two phenomena is not clear at present. For example, flux studies in well-characterized porous membranes show that Ficoll molecules display a higher rate of flux through cylindrical pores than do dextrans with the same free diffusion coefficients. Put differently, a Ficoll fraction will have a larger Stokes radius than a dextran fraction with the same rate of restricted diffusion. This effect is explained by the larger contribution of the distal segment density to the steric hindrance of the polymer in the pore than to the friction coefficient (Anderson, J., personal communication). On the other hand, the available SEC data show virtual congruence of K_{SEC} vs R_η for dextran and amylose vs pullulan, along with the linear water-soluble polymer poly(ethyleneoxide) (Kuge *et al.*, 1984); for pullulan and dextran (Kato *et al.*, 1983); and for pullulan vs Ficoll (Hussain *et al.*, 1991); suggesting that permeation in SEC only depends on the hydrodynamic size in free solution. But it is important to note that the polydispersity of one or more of the samples employed in these studies can easily obfuscate such conclusions.

In this paper we review the behavior of a number of neutral polysaccharides on various SEC packings. The key variable is the chromatographic partition coefficient

$$K_{SEC} = (V_e - V_0)/(V_t - V_0), \quad (1)$$

where V_e is the measured elution volume of the solute of interest, V_0 is the interstitial volume (between packing particles), measured as the retention volume of a very large solute, and V_t is the total liquid volume in the column, measured as the retention of e.g. D_2O or glucose. Thus, $V_t - V_0 = V_p$, the pore volume. K_{SEC} thus represents the fraction of the pore volume to which the solute has effective access. We discuss the evidence that K_{SEC} of neutral polysaccharides is governed by entropic factors only, i.e. without adsorption. Comparisons between the chromatographic behavior of polysaccharides and proteins will be discussed. Recent evidence that suggests that the SEC behavior of Ficoll is representative of the chromatography of impenetrable spheres will be presented. The implications of measurements made on a rod-like polysaccharide, schizophyllan, will be reviewed in detail.

SEC TECHNOLOGY

A SEC system differs from other HPLC systems only in column packing and detection devices; the pump, injector and electronic controllers are identical to those of an isocratic HPLC. Column packings for SEC can be considered as of two types: those made from either synthetic or partly natural polymers, and those made from inorganic solids, namely glass or silica. Prior to 1970, all aqueous SEC packings of the former type were 'soft' gels, e.g. Sephadex, while the latter were made of porous glass beads. Neither was very satisfactory. The 'soft' polyacrylamide- or dextran-based gels were too compressible to be used under pump pressure, and were generally used under gravity flow. Separations were low-efficiency (wide peaks) and slow (one or more hours). Porous glass packings could withstand high pressure, but were also incapable of maintaining compact peaks, and furthermore were highly adsorptive to proteins (always a major concern to producers of aqueous SEC columns). Two significant technological developments took place in the 1970s. (1) Small particle-size, semi-rigid cross-linked hydrophilic polymer gels were introduced by Toyo Soda company, and marketed under the name 'PW gel'. These allowed for relatively non-adsorptive, fast protein chromatography. (2) Small particle-size porous silica, previously used for normal-phase HPLC, was modified in two ways: much greater control of porosity was achieved, and surface derivatization, using silane chemistry, was used to reduce the adsorptivity of these packings with respect to proteins. Products based on this technology were offered by Toyo Soda, Waters Assoc., Brownlee Labs, Synchrom, and others. Both types of materials, along with concomitant developments in packing methods, yield columns with efficiencies in excess of 10 000 plates/meter, so that high resolution in short times is possible. For the protein chemist, the significance of high resolution is the ability to separate and analyze complex multicomponent samples; for the polymer chemist, the accurate assessment of the molecular weight distribution is the significant consequence of high efficiency. Speed of analysis is always important, but particularly in industrial analytical and QA/QC environments.

Numerous suppliers offer surface-modified porous silica columns for aqueous SEC. The diversity of materials is greater for packings made from synthetic or natural polymers. Semi-rigid stationary phases based on cross-linked hydrophilic synthetic polymers are supplied by Hitachi, Polymer Labs, Bio-Rad, LKB, Showa Denko and Asahi, along with the Toyo Soda columns mentioned above. Packings based on cross-linked polysaccharides are generally low-efficiency and non-rigid, with the exception of the cross-linked agarose material supplied by Pharmacia as Superose. Columns packed with porous glass- or silica-based materials are produced by Waters Assoc., E. Merck, DuPont, Electron-Nucleonics, Toyo Soda, Rhone-Poulenc and Synchrom. Details on these different packings may be found in the literature (Unger, 1988).

The second aspect that differentiates SEC systems from other HPLC arises from the fact that the properties of polymers lead both to different questions about sample characterization and different detection capabilities. These particularly take advantage of the enhancement of light scattering and of viscosity by dilute polymer solutions. Light scattering detectors are usually based on total intensity measurements ('static' light scattering) which can lead to a measurement of the mass and radius of gyration of the solute. By using high intensity lasers and thus very low solute concentration, and by measuring at low scattering angle, Chromatix was able obviate the need for determination of angular and concentration dependences synonymous with Zimm plots, and thus provide the first SEC molecular weight detector. Wyatt Technology has subsequently designed a light scattering instrument with a flow cell, which in principle, can provide more information about the sample. The second light scattering technique of interest is quasielastic light scattering, also known as photon counting spectroscopy or dynamic light scattering. From this method one obtains the diffusion coefficient and thence the Stokes radius. A dynamic light scattering detector was developed by Oros and is now marketed by Biotage. Viscosity detectors do not yield absolute molecular weights, but do provide information about polymer size and shape. Viscotek is the principal source of such devices.

FLEXIBLE CHAIN NEUTRAL POLYSACCHARIDES

Dextran is an extracellular polysaccharide from *Leuconostoc mesenteroides (B-512)*. The structure of this (1 → 6)-linked α-D-glucan is shown in Fig. 1. Dextran has long been used as the MW calibration standard for aqueous SEC. The principle drawback until recently was that the MW samples available from Pharmacia (e.g. T-10, T-40, T-70) are rather polydisperse, with M_w/M_n typically around 1.5–2. Furthermore, the branching state is complex: dextran has both short- and long-chain branches, and the distribution of these is MW-dependent. Thus, at MW > 10^6, a marked reduction in the dependence of the intrinsic viscosity on MW, corresponding to a decrease in the Mark–Houwink exponent from *c.* 0.5 to 0.2 (Kuga, 1981), shows the large effect of long-chain branching (LCB) at high MW. A more quantitative analysis of the LCB content of dextrans was carried out on the basis of light scattering, viscometry and SEC results (Kuge *et al.*, 1987). Narrow MWD dextran samples, characterized by low angle light scattering, viscometry, reducing end-group titration, and SEC, are now available from Pharmacosmos (Viby Sj., Denmark).

Pullulan, an extracellular polysaccharide from the fungus *Aureobasidium pullulans*, may be viewed as maltotriose coupled via α-1,6-glucosidic linkages. Its basic molecular characteristics have been described in detail (Kato *et al.*, 1982; Kawahara *et al.*, 1984), and its dimensional and hydrodynamic properties have been studied thoroughly (Kato *et al.*, 1984; Buliga & Brant, 1987). Narrow MWD fractions of pullulan (M_w/M_n < 1.2) are commercially available from Show Denko.

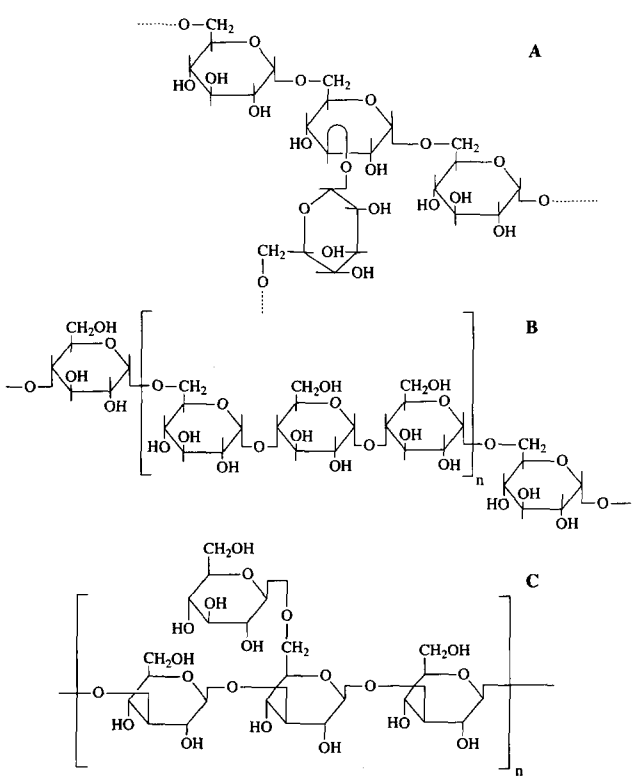

Fig. 1. Repeat unit structures of nonionic polysaccharides used in size exclusion chromatography studies: (A) dextran; (B) pullulan; (C) schizophyllan.

NON-RANDOM COIL NEUTRAL POLYSACCHARIDES

Early research at Purdue on amylose yielded the first evidence for helical configuration in a biopolymer. Since that time many polysaccharides have been found to form single and multichain helices. Some of the multichain helical configurations stable in aqueous solution are those of xanthan and carrageenan. Most of these are anionic, and schizophyllan, formed from *Schizophyllum commune* (Kikumoto *et al.*, 1970), is one of the few stable helical nonionic polysaccharides. Schizophyllan is formed from linearly linked (1 → 3)-β-D-glucose residues with (1 → 6)-β-D-glucose side chains. In water, it forms a rigid triple helix (Norisuye *et al.*, 1980). In a manner characteristic of uncharged rigid polymers, schizophyllan displays a pronounced tendency towards aggregation, and towards the formation of a liquid crystalline phase (Itou & Teramoto, 1984). However, under suitable solution conditions, the properties of isolated schizophyllan molecules in solution have been examined in considerable detail (Kashiwagi *et al.*, 1981). The conclusion of these studies is that the triple helix is stable over a wide range of conditions. The pitch is 0.30 nm per glucose residue, and the persistence length is 180 ± 30 nm, from which it may be concluded that high-MW schizophyllan is a semiflexible rod. For MW < 3 × 10^5, the exponent in the Mark–Houwink equation $[\eta] = KM^a$ is a = 1.8, which is the theoretical value for a rigid rod (Kashiwagi *et al.*, 1981).

In contrast to the materials noted above, Ficoll is a synthetic polysaccharide, formed by copolymerization of sucrose and epichlorohydrin (Holter & Møller, 1956). Ficoll is of special interest in the evaluation of permeability of biological membranes since it resembles proteins more closely than does dextran in regard to compactness. Naturally, a model for the permeability of membranes to proteins that only takes into account size and compactness is extremely simplified; even so, one may hope that the steric contributions to membrane permeability might to some extent be discerned by means of studies with a 'globular' macromolecule that does not participate in any specific interactions with the membrane. With regard to size exclusion, the relatively compact symmetric structure of Ficoll makes it a more useful solute for testing models of separation mechanism.

EVALUATIONS OF SEC MODELS WITH NONIONIC POLYSACCHARIDES

Although SEC has been in use for more than 30 years, the mechanism of separation is still a matter of debate. The early years saw the development of two groups of models for SEC, those based on flow, and those entailing only entropic contributions to the equilibrium between the interstitial fluid and the liquid entrapped in the non-mobile domains of the gel. Despite the intuitive appeal of separation involving flow, such models have been generally considered inconsistent with the marked insensitivity of K_{SEC} to flow rate. It is also generally perceived that batch equilibrium of solution with stationary phase gives rise to partitioning coefficients identical to the chromatographic values, but careful measurements along these lines are in fact very few. Thus dynamic mechanisms for separation have only gradually been discarded during the last two decades. Even so, considerable debate exists over an appropriate model for the equilibirium partitioning coefficient. An early approach taken by Ackers treats the range of possible retention times as a direct consequence of the pore size distribution (PSD). Assuming that this distribution is the error function, Ackers obtained (Ackers, 1970):

$$K_{SEC} = 1 - \mathrm{erf}[(R - a_0)/b_0], \tag{2}$$

where, in a gel with identifiable pore geometry, a_0 would be the average pore radius, and b_0 would be the standard deviation of the pore distribution. While this approach ingenuously avoids the problem posed by the unknown geometry of the substrate pores, it is wrong because it fails to consider the partial permeation of solutes into pores, assuming instead that a given solute is totally excluded from some fraction of the pores (true) and has complete access to the remaining pore volume (false). The model wrongly predicts that the calibration curve for an extremely narrow PSD substrate would exhibit only two elution volumes, and this is not the case. Despite this evident failure, the Ackers treatment is still cited in the biochemical literature (Cantor & Schimmel, 1980).

An approach which does model the pore is that of Laurent and Killander (1964), based on the Ogston treatment for the centrifugal velocity of a sphere in a suspension of rods (Ogston, 1958). This approach gives rise to the following relation:

$$K_{av} = \exp[-\pi l(R + r_f)^2], \tag{3}$$

where r_f is the cross-sectional radius of the fibers that constitute the gel, and l is the linear concentration of fibers in the gel. K_{av} is the probability that a spherical particle is included in the network of the gel and is related to K_{SEC} by the expression

$$K_{av} = K_{SEC} V_i/(V_i + V_m), \tag{4}$$

where V_i is the total pore volume and V_m is the volume of the gel matrix (nonporous portion of the gel). In the case of agarose gels, for example, a system of randomly intersecting rodlike fibers might conceivably be a reasonable model for the gel. However, the actual permeation in the large domains accessible to typical solutes might sample a collection of cavities which are not well-represented by this model.

The simplest model proposed for equilibrium separation in SEC is based solely on geometric considerations. The resulting expression is:

$$K_{SEC} = (1 - R/r_p)^{\lambda}, \tag{5}$$

where $\lambda = 2$ for cylindrical pores, and 1 or 3 for slab or spherical pores (Casassa, 1971). This model has been used most extensively by Waldmann-Meyer (1985).

A more generalized model was proposed by Giddings, in which the substrate was modeled as a collection of randomly intersecting planes, and the solute as capsules with various aspect ratios. In this case the porosity of the gel appears as the surface to volume ratio, as in:

$$K_{SEC} = \exp(-s\bar{X}), \tag{6}$$

where s is the pore surface area per unit of free volume, and X is half of the mean projection length of the solute (Giddings *et al.*, 1968).

The foregoing section summarizes the principal models put forward to explain the migration of rigid, compact solutes in SEC columns. One notes that the forms of these expressions are apparently quite different. In the Ackers model there is a linear relationship between the solute radius and the inverse error function of K_{SEC}; the Laurent and Killander model has the Stokes radius varying with the square root of $-\ln K_{av}$; and the Waldmann-Meyer treatment predicts a linear dependence of the hydrodynamic radius on $1 - K_{SEC}^{1/2}$.

At this junction, one might ask why such conflicting models would co-exist for 20 years or more, and not be quickly reduced in number by experimental test. The answer has three parts. First, validation of these models has often been reported simply on the basis of linear fits according to the foregoing dependences. We have recently shown that for mathematical reasons alone, $\mathrm{erf}^{-1}K$, $[-\ln K]^{1/2}$, and $1 - K^{1/2}$ are all co-linear over relatively wide ranges of K; in other words, the true tests of validity require good data at extreme values of K. Furthermore, good fits may be obtained using quite unrealistic values for the fitting parameters in eqns (2) and (3).

A second problem is that the preceding expressions can only be tested by solutes of well-defined geometry, preferably spheres. Ordinarily, proteins have been used to test these expressions, and these solutes are approximated by ellipsoids of variable axial ratios. A more serious problem concerning the use of proteins to test SEC models is the likelihood of interactions with the packing. Nearly all aqueous SEC packings bear some

negative surface charge (Dubin, 1988). Substrates based on glass or silica carry a charge arising from dissociated silanol groups, while polymer-based packings, such as Superose or PW gel, bear carboxylate groups. Because of the abundance of silanol groups on siliceous packings, those stationary phases have much larger surface charge densities and therefore adsorb proteins via electrostatic interactions more strongly. Most siliceous packings used for SEC of proteins have a covalently bound hydrophilic layer. However, as pointed out by Iler (1981), even with the smallest silane moiety one can at at best derivatize half of the silanol groups. Therefore, the majority of SiO^- groups are preserved; they may be 'buried' but their electrical field remains influential. pH adjustment can be used to diminish the electrostatic interaction between protein and stationary phase, but since a pH change that reduces the positive protein charge will increase the negative packing charge, and vice versa, it is not clear that any pH will eliminate the coulombic interaction. At sufficiently high pH, repulsive effects are likely to contribute to K_{SEC}. Coulombic interactions are probably very weak at ionic strengths above 0.5, but here a diminution of protein solubility is likely to promote hydrophobic interactions with the column. Thus it is likely that the 'scatter' commonly observed in calibration curves of R_S vs K_{SEC} for proteins reflects in part the variable interactions between solutes and packing.

Flexible chain polysaccharides have been frequently used to test theories of SEC migration. Using data for dextrans on porous glass, Haller *et al.* (1977) justified a semi-empirical expression of the form

$$K_{SEC} = 1 - (R/r_p)^N, \tag{7}$$

where R and r_p are effective dimensions of the solute and the pore, respectively, and N is an empirical pore-size-dependent variable, given by

$$N = a(r_p)^b, \tag{8}$$

A similar relationship was proposed by Waldmann-Meyer (1985):

$$K_{SEC} = (b - R/r_p)^2, \tag{9}$$

where b is found to be slightly larger than unity because "small solvent molecules can diffuse into the glass" (*sic*). Since the 'equivalent sphere radius' for dextrans in water is roughly proportional to the square root of the molecular weight, Waldmann-Meyer verified the preceding relationship by plotting $K_{sec}^{1/2}$ vs $MW^{1/2}$ and derived support for eqn (7) from the linearity of these plots for dextran fractions on CPG porous glass. Poitevin and Wahl (1988) measured the chromatographic partition coefficient on Sephadex for fluorescein-labeled dextran fractions, using fluorescence recovery after photobleaching (FRAP). From the dependence of $\ln K_{av}$ on the Stokes radius of dextran, R_S, they established the accuracy of eqn (3). However, it is worth pointing out the same data of Poitevin and Wahl give equally compelling fits when plotted according to eqns (2) or (5) (Unger, 1988). Similarly, the data used by Haller to verify eqn (7) lend compelling support for eqns (2), (5) or (8). As noted by Unger (1988) one of the reasons for these seemingly ambiguous observations is the fact that all the foregoing functions tend to be co-linear in the region of intermediate K, where data are commonly collected. A second reason is that simple goodness of fit is not an adequate criterion; one must look for realistic values of adjustable parameters, such as r_f, r_p or a_0. The third consideration is that all of the foregoing models treat a hypothetical spherical solute. *A priori*, there is no reason to assume that a flexible chain polymer such as dextran has the same migration velocity in an SEC column as a hard sphere with equal free diffusivity; yet this is what is assumed when R_S is substituted into eqns (2), (3), (5) or (9). In order to resolve this last difficulty, we turn to compact spherical solutes.

SEC OF FICOLL

Figure 2 shows the dependence of K_{SEC} on solute size for Ficoll fractions and pullulan, on a Superose 12 column. Here, the solute size is characterized by the viscosity radius (Flory, 1953):

$$R_\eta = \{3[\eta]M/4\pi(0.025)N_A\}^{1/3}, \tag{10}$$

where the units of R_η are in cm when the units of $[\eta]$, the intrinsic viscosity, are in dl/g. The Stokes radius

$$R_S(\text{Å} = kT/(6\pi\eta D_0)10^{22}, \tag{11}$$

(where D_0 is the translational diffusion coefficient in $cm^2\ s^{-1}$, and η the solvent viscosity, i.e. 0.0010 P for water) has been used frequently to calibrate SEC for proteins; however, the majority of accumulated data suggest that the viscosity radius provides greater congruence of plots for different macromolecular shapes (Frigon *et al.*, 1983; Potschka, 1987). The pullulan fractions are of narrow MWD, so the value of K_{SEC} and the viscosity radius can all be considered to correspond to the same molecules. However, the MW polydispersity of the Ficoll fractions is as large as $\bar{M}_w/\bar{M}_n = 1.9$. In this case, the component eluting at the chromatographic peak is probably of lower MW than the component that has the same intrinsic viscosity as the bulk sample. Thus, substitution of M from light scattering and the measured $[\eta]$ into eqn (10), and plotting vs K_{SEC} from the chromatographic peak is mixing three different moments of the distribution. In Fig. 2 this problem is solved by normalizing the values of M and K to the z-average (third moment) of the distribution. The solid curve drawn through the data is the theoretical curve, which is based on a model of the gel as a collection of cylindrical pores, each of which obeys eqn (5), with a distribution of pore sizes given by an

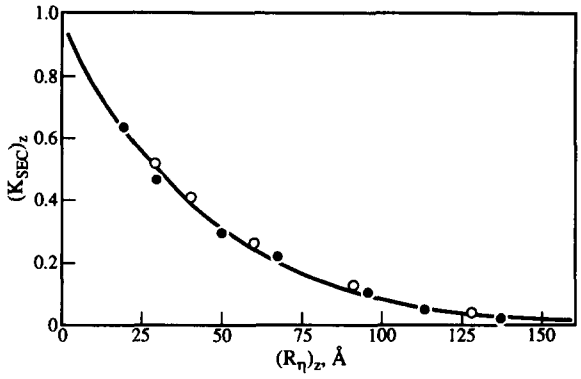

Fig. 2. Dependence of chromatographic partition coefficient on the viscosity radius for Ficoll (●) and pullulan (○) on Superose 12, in 0.38 M, pH 5.5 phosphate buffer. Solid line is the theoretical curve (see text for explanation). Both K_{SEC} and the viscosity radius have been corrected to correspond to the *z*-average moment of the distribution. From Hussain *et al.* (1991).

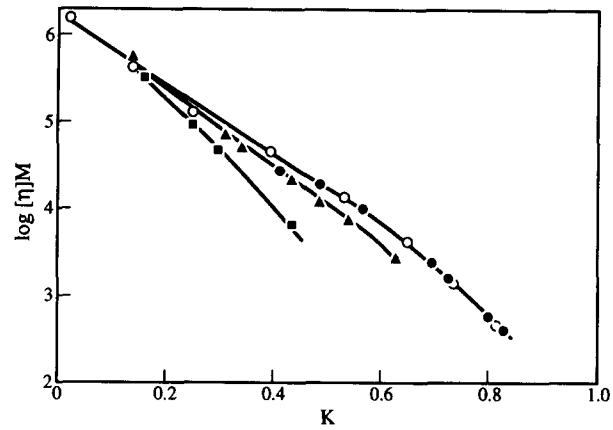

Fig. 3. Dependence of chromatographic partition coefficient on the 'universal calibration parameter' $[\eta]M$, for proteins (●), pullulan (○), DNA (▲) and schizophyllan (■), on Superose-6, in pH 5.5, ionic strength 0.38 M phosphate buffer (from Dubin & Principi, 1989). Note that, since $[\eta]M \sim (R_\eta)^3$, $\log[\eta]M \sim \log R_\eta$.

error function. The mean pore size is obtained as 120 Å by fitting, and the distribution of pore sizes is consistent with reports from Hagel (1988). The important conclusions are (a) that an expression based on modeling the gel as a collection of cylindrical pores, yields, with reasonable values for pore size and pore distribution, a dependence of retention time on size as observed for non-interacting spheres; and (b) the viscosity radius appears to govern the retention of compact spheres and flexible chains (measured values of K_{SEC} vs R_S for Ficoll could not be brought into congruence with the theoretical curve with any choice of pore size and distribution). This last finding is in accord with results for dextran and proteins (Frigon *et al.*, 1983) and pullulan and proteins (Dubin & Principi, 1989), although it should be noted that proteins never conform precisely to a smooth curve. Figure 2 shows less congruence of the data when the Stokes radius is used. While the scatter precludes any definitive assertion, the results may suggest that the most compact solute used in this particular study (dendrimers) does exhibit larger K_{SEC} than the polysaccharides of equal Stokes radius, and this observation is in accord with results from hindered diffusion studies (Anderson, J., personal communication).

SEC OF SCHIZOPHYLLAN

The preceding results, as well as those obtained for numerous studies involving various flexible chain linear polymers, all indicate 'universal calibration' when the viscosity radius is the independent variable. The only clear-cut exception is provided by results obtained for schizophyllan (Dubin & Principi, 1989) on Superose 6. As shown in Fig. 3, schizophyllan elutes earlier than a flexible chain polymer (pullulan) or globular proteins of equal R_η. A similar, but less obvious, trend is seen for DNA. This result conflicts with findings of Potschka for DNA vs proteins (Potschka, 1987), but in that case, the DNA samples were all well above a MW corresponding to the persistence length of DNA, *c.* 50 nm. In Fig. 3, the DNA samples are all less than 500 base pairs, and therefore more rigid. With a persistence length of *c.* 120 nm, schizophyllan is more rigid and such effects persist to higher MW than for DNA.

A phenomenological approach to the behavior of rod-like polymers in SEC (Dubin *et al.*, 1990) may be developed by assuming some relationship between a measured dimension, e.g. R_η, and the hypothetical dimension that controls SEC, which we call R_{SEC}. For a rod of diameter d, we propose:

$$R_{SEC} = \alpha R_\eta [d/2R_\eta]^\beta. \tag{12}$$

For spherical solutes, $d = 2R_\eta$, $\alpha = 1$ and $\beta = 0$. Thus R_{SEC} may be thought of as the size of the 'equivalent sphere' which has the same migration velocity as the solute in question (whose viscosity radius is R_η). If we assume that the geometric equation (eqn 5) is satisfied by R_{SEC}, then

$$K_{SEC} = [1 - 1/r_p\{\alpha R_\eta (d/R_\eta)^\beta]^\lambda. \tag{13}$$

Rearranging,

$$-\ln(1 - K_{SEC}^{1/\lambda}) = \ln(r_p/\alpha) - \beta \ln d + (\beta - 1)\ln R_\eta, \tag{14}$$

Thus, a plot of the lns of eqn 14 vs ln R_η should be a straight line with a slope of $\beta - 1$. Such plots are shown in Fig. 4, along with data for globular proteins and DNA fractions.

Table 1 shows the results of these plots in terms of the values of β and the correlation coefficients. The correlation coefficients are all remarkably close to unity, indicating the excellent fit of the data. The value of the

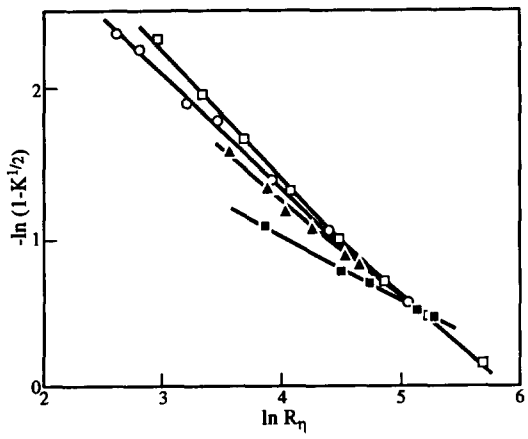

Fig. 4. Data from Fig. 3, plotted according to eqn (13) with $\lambda = 2$: pullulan (□), proteins (○), DNA (▲) and schizophyllan (■). From Dubin *et al.* (1990).

Table 1. Fit of SEC data to eqn 13

	Pullulan	Proteins	DNA	Schizophyllan
β	0.18	0.23	0.36	0.57
r	0.998	1.00	0.996	1.00

empirical parameter β is seen to increase with solute asymmetry. It is interesting to note that its value is slightly different for pullulans and proteins, and zero for neither. This may suggest that the application of eqn 13 to Superose gels is incorrect, but an intriguing possibility is that this method can be used to discern some level of either permanent or transient deviation from spheroidal symmetry for both proteins and Gaussian chains.

CONCLUSIONS

Nonionic polysaccharides display no evidence of specific interactions with any of the stationary phases used in aqueous size exclusion chromatography. In contrast to the SEC behavior of globular proteins, for example, their elution rates may be assumed to be governed by steric effects alone. For this reason, they are optimal materials for testing theories put forward about size exclusion. Most theories model the solutes as spheres, and Ficoll, a compact symmetrical polysaccharide, is the most appropriate solute for testing such theories. Pullulan provides a good paradigm for the SEC of flexible chain polymers. Studies on schizophyllan, a stiff triple-helical polysaccharide, help to elucidate the effective SEC dimension of asymmetric or rod-like solutes.

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