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Permeation of Small Molecules in Aqueous Size-Exclusion Chromatography Vis-à-Vis Models for Separation

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K_{SEC}, the chromatographic partition coefficient for size-exclusion chromatography, is defined as the fraction of the column pore volume into which a solute can permeate. The column pore volume is commonly measured as the elution volume of a small molecule, e.g., D_2O in the case of aqueous separations. We found that the elution volumes of a number of small molecules vary inversely with molecular size, so that the choice of any single small molecule is arbitrary. For a large number of small molecules and oligomers, we found two distinct regions in the dependence of $K_{SEC}^{1/2}$ on solute size R, with a discontinuity at $R \cong 0.6$ nm. These results are explained in terms of two sets of pores: (a) those accessible to all solutes, which behave to a first approximation as cylindrical cavities, and (b) micropores, accessible only to solutes smaller than 0.6 nm. If K_{SEC} is calculated by using an adjusted pore volume, reduced by the 20% attributable to micropores, the data for the larger solutes are found to conform to the cylindrical pore model: $K_{SEC} = (1 - R/r_p)^2$.

A central problem in size-exclusion chromatography (SEC) is the absence of a fundamental relationship between the retention time and the dimensions of the solute and the stationary-phase pores. In practice, the problem is addressed by column calibration in which the relationship between elution volume (V_e) and molecular size (or more typically, molecular weight, MW) is established with well-characterized standards. This procedure is lacking for several reasons: (1) The dependence of V_e on molecular weight should not be uniform unless the compounds all have the same relationship between molecular dimensions and molecular weight. (2) When the relationship between migration velocity and solute dimensions is purely empirical, column calibration depends on an arbitrary selection among various plotting procedures; if the correct functional form of V_e(MW) is unknown, it is more difficult to identify experimental error or distortions arising from nonideal behavior (solute−stationary phase interactions). (3) Because the relationship between solute dimensions and migration velocity is not well understood, the nature of the solute size determined by SEC is unknown.

Some of these problems were addressed by "universal calibration",[1] which was based on the finding that linear and branched random coil polymers all eluted according to their hydrodynamic radius

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

where R_η has units of centimeters when $[\eta]$ is the intrinsic viscosity (in dL/g). The universality of this principle among macromolecules of varying asymmetry, i.e., spheres and rods, is controversial.[2] Universal calibration then only partly addresses item 2 above. Thus, when column calibration with a set of well-characterized proteins produces "scatter", it is difficult to know whether these deviations arise from varying axial ratios, differences in protein-specific solute−gel interactions, or indeed experimental error.

Solute migration in SEC is generally reported as the chromatographic partition coefficient:

$$K_{SEC} = (V_e - V_0)/(V_t - V_0) \quad (2)$$

V_e is the solute elution volume; V_0, the interstitial column volume, is taken as V_e for a fully excluded molecule; and V_t, the sum of V_0 and the pore volume, is obtained as the elution volume of a small molecule. K_{SEC} is generally assumed identical to the equilibrium partition coefficient and thus also represents the relative probability of finding the solute in the pore, i.e., the concentration of solute within the pore relative to the mobile phase.[3,4] The desired relationship is then

$$K_{SEC} = f(R,r_p) \quad (3)$$

where R and r_p describe the dimensions of the solute and pore, respectively.

Concerning the function $f(R,r_p)$, many suggestions have been made. Laurent and Killander[5] explained the retention of proteins on Sephadex G-200 via the theory of Ogston,[6] which models the

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gel as a uniform suspension of fibers. However, the cavities that emerge from this model might not resemble the larger domains actually sampled by typical solutes. Giddings[7] formulated a statistical mechanical expression for the partitioning of solutes into a matrix of randomly intersecting planes. K_{SEC} was expressed in terms of the ratio of the solute's mean external length (only the distal groups affect partitioning) to the surface area per unit free volume of the matrix (reciprocal hydraulic radius). Giddings noted that a surface roughness correction would be necessary if the probe molecule that measures the surface area "sees" a surface different from the partitioning macromolecule. leMaire[8] accounted for surface roughness by treating the pore as a fractal with dimension ranging from 2 (smooth surface) to 3 (highly irregular). Ackers[9] completely avoided pore descriptions, assuming that any solute is totally excluded from some pores (true), with complete access to the others (false).

These analyses focus on the pore description and assume simple solute geometry (capsules in one case,[9] but generally spheres). Casassa,[3] however, considered random coil polymer solutes and developed a statistical mechanical treatment for the depletion layer inside the pore. The result was consistent with universal calibration for linear and branched random coils but indicated divergent behavior for rods and spheres.[10] For simplified pore geometries, it was observed that spherical solutes of radius R should follow the relation

$$K_{SEC} = (1 - R/r_p)^{\lambda} \qquad (4)$$

where r_p is the dimension for slab ($\lambda = 1$), cylindrical ($\lambda = 2$), and spherical ($\lambda = 3$) pores. Waldmann-Meyer,[11] using hydrodynamic radii for R, reported very good agreement with eq 2, $\lambda = 2$, for flexible-chain polymers on porous glass packings. Dubin[12] modified this approach by proposing a Gaussian distribution of cylindrical pores to fit data for the retention of densely branched Ficolls on Superose packings.

There are several reasons that such apparently conflicting theories have coexisted over decades. First, the value of R is ambiguous, apart from the few solutes that may approximate spheres. The situation is even worse for r_p, since the structure of the pores in commercial SEC packings is quite irregular. The pore dimension is usually obtained by fitting the data to a model based on, for example, a random array of rodlike fibers, a uniform set (or distribution) of cylinders, an assembly of intersecting planes, or a fractal solid. This is generally done without much comparison to real stationary-phase structure, although the apparent pore radius from SEC via the cylindrical pore model usually agrees with the results of mercury porosimetry (restricted to rigid packings). Second, the testing of theoretical expressions requires solutes that not only have simple geometry but also do not interact with the packing. This is particularly problematic in aqueous SEC. Related to this is the question of experimental precision and the willingness to accept deviations from the theoretical expectation of up to 10% as experimental error. Last, SEC data show a remarkable tendency to conform to several theories, because the various $K_{SEC}(R)$ functions tend to be collinear in the range of $0.2 < K_{SEC} < 0.8$ where measurements are commonly made.[12]

This paper addresses an additional and generally neglected problem: the determination of V_t, upon which the value of K_{SEC} depends. The small molecule used to measure V_t is typically considered to probe all of the space *accessible to large solutes*. Thus, the diminution of solute concentration within a pore is considered to arise only from the exclusion of *the center of mass* of the solute from the proximity of the pore walls. This, however, would be incorrect if the pore contained microfissures. In this situation, the choice of the low molecular weight solute becomes a critical one: should it probe all solvent-accessible volume, or should regions accessible to solvent alone be eliminated from this measurement? Even in the absence of complex pore structure, selection of the low molecular weight probe is a problem, because V_e is unlikely to be truly independent of solute size. Indeed, an extensive body of work demonstrates the feasibility of separating small molecules by SEC.[13] Nevertheless, low molecular weight probes of V_t are commonly assumed to be somewhat interchangeable and arbitrarily assigned zero solute size, corresponding to $K = 1$. This approximation becomes more tenuous as phase pore size diminishes.

We report here on the size dependence of the retention of noninteracting low molecular weight solutes. We selected Superose 12, which provides high resolution in the low molecular weight range, coupled with column stability and minimal interactions with uncharged solutes.[14] The solutes chosen were oligomers of poly(ethylene oxide), oligomers of dextran,[15] and a number of diols and other hydrophilic solutes. For solutes with molecular weight in excess of 1000, dimensions could be determined as equivalent hydrodynamic radii, either from viscometry via eq 1 or from diffusion coefficients, via the Stokes−Einstein relation,[16] referred to henceforth as R_η and R_S, respectively. For lower molecular weight solutes, dimensions were obtained by molecular modeling. It is important to note that both computer modeling and experimental dimensions were accessible for solutes in the range of $R \cong 1$ nm.

EXPERIMENTAL SECTION

Materials. Low molecular weight solutes and solvents were from Aldrich or Sigma. PEO MW 200−8000 were gifts from Dow Chemical Corp., and PEO of MW 14 000 or higher was from Aldrich. Oligodextrans were prepared by SEC preparative fractionation.[15]

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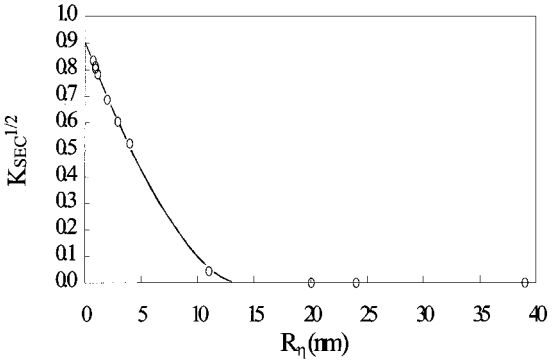

Figure 1. Dependence of chromatographic partition coefficient on viscosity radius for PEO, MW > 500.

SEC. Chromatography was carried out on a Superose 12 column (Pharmacia LKB, Uppsala, Sweden), coupled to a Milton Roy (Riviera Beach, FL) minipump, and a Waters R401 differential refractometer. The injection (Rheodyne) volume was 20 μL, and the mobile phase was type 1 reagent grade water. Eluant concentrations were 2−4 mg/mL for PEO, 5 mg/mL for D_2O, and 1 mg/mL for all other solutes. All solutions were filtered through 0.22-μm Nylon (MSI, Westboro, MA) filters prior to injections. Elution was carried out at 0.5 mL/min. In addition, retention volumes of D_2O and 600K PEO were measured at 0.25 mL/min and found to be identical within 1%, indicating that both these flow rates were too low to give rise to nonequilibrium partitioning. V_0 and V_t were always determined along with V_e to ensure consistency in the measurement of K_{SEC}. Flow rates were measured gravimetrically, with a precision of ±0.5% or better, during and at the beginning and end of each elution sequence.

QELS. Stokes radii for several low molecular weight polymers were measured by quasielastic light scattering, using a Dynapro model 801(Protein Solutions Inc., Charlottesville, VA), which employs a 30-mW solid-state 780-nm laser and an avalanche photodiode detector. Samples were introduced into the 7-μL cell via 0.1-μm Anotec filters, and the 90° scattering data were analyzed via the method of cumulants. The measured diffusion constant was used to obtain the apparent Stokes radius, R_S. This analysis is based on the assumption that the mutual diffusion coefficient can be equated to the translational diffusion coefficient, which presupposes that contributions from interparticle interactions are small, a good assumption for the nonionic solutes of this study.

Molecular Modeling. Molecular modeling of small molecules and oligomers was carried out with QANTA (Molecular Simulations, Inc., Burlington, MA), using the CHARMm energy functions to minimize potential energy. With H_2O as the probe molecule, we then calculated the solvent-accessible surface area (SAS) for each molecule, as the area defined by the center of the probe when it rolls over the molecular surface. The corresponding molecular radius R_{SAS} was then obtained by assuming spherical molecular geometry.

RESULTS AND DISCUSSION

Figure 1 shows a plot of the chromatographic partition coefficient, calculated according to eq 2, in which the pore volume ($V_p = V_t - V_0$) is—provisionally—obtained using the elution volume of D_2O as V_t. The data for ethylene oxide polymers of MW ≥600 are plotted according to the cylindrical model of eq 4, using values for R_η values reported elsewhere.[17,18] The intercept of 0.90 at $R = 0$ is, however, significantly smaller than the expected value of unity. While there is some question about the form of the extrapolation to $R = 0$, given the likelihood of pore size distribution,[19] the modest curvature of the data for larger solutes (see inset of Figure 2) make it difficult to imagine any reasonable extrapolation to $K = 1$ at $R = 0$. This result suggests that the effective pore volume for PEO, V'_p, which should be the denominator in eq 2, is smaller than the experimental value of 13.78 mL obtained using D_2O and MW 6×10^5 PEO. The discrepancy between V_p and V'_p could arise from an additional volume accessible to small molecules but not to larger ones.

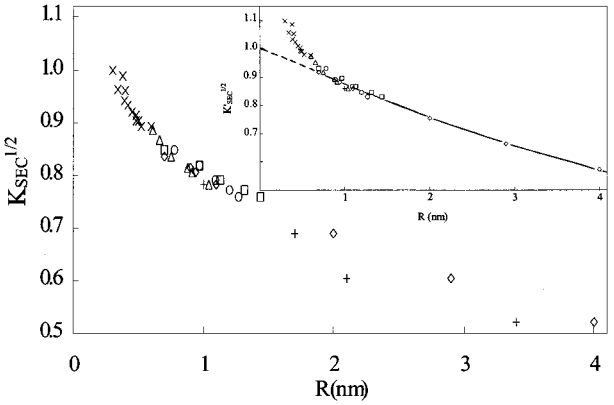

Figure 2. Chromatographic partition coefficient for the solutes of this study as a function of solute radii measured in various ways: (○) oligodextrans, R_{SAS}; (□) oligodextrans, R_η; (X) low molecular weight solvents and solutes, R_{SAS}; (◇) PEO, R_η; (△) PEO, R_{SAS}; (+) PEO, R_S. Inset: R dependence of $(K'_{SEC})^{1/2}$, where K'_{SEC} is the partition coefficient on a hypothetical micropore-free column. For higher molecular weight PEO, only R_η was used because this quantity was most uniformly available.

To examine in more detail this proposed explanation, we eluted a series of small molecules and oligomers, described in Table 1 and represented by the various symbols in Figure 2, which identify both the nature of the solute and the technique used to determine its size. Included among these were two additional oligomers of PEO, namely, of nominal MW 200 and 300. (The proximity of these nominal values to the true mean molecular weight was confirmed by mass spectrometry.) For these various oligomers and small molecules, direct determination of intrinsic viscosities are not feasible, and other methods for determination of molecular dimensions were required. For the two PEO oligomers, MW 200 and 300, Mark−Houwink constants of K and a, 0.156 mL/g and 0.5, respectively, obtained for PEO in this low molecular weight range[20] were used to calculate $[\eta] = KM^a$ and, subsequently, R_η via eq 1. R_η for oligodextrans was obtained either by using K and a values of 0.0493 mL/g and 0.6, respectively, reported to be valid for the MW range 2000−45 000,[21] or by extrapolation from

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(18) Reference 17 provides a summary of "hydrodynamic radii" (R_h) for PEO. While these data are based on both viscosities and diffusion coefficients, and also appear to correspond to various temperatures, the collected results correlate very well with a smooth function of log R_h vs log M; the values reported in Table 2 are interpolated from this plot.

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Table 1. Computed and Measured Properties of Solutes[a]

	MW	$K_{SEC}^{1/2}$	R_{SAS} (nm)
	Small Molecules		
D_2O	20	1.00	0.30
MeOH	32	0.96	0.34
acetonitrile	41	0.99	0.38
acetone	58	0.96	0.40
glucose	180	0.90	0.48
sucrose	341	0.89	0.60
EO	62	0.94	0.39
DEO	106	0.92	0.45
TEO	150	0.90	0.50
2ME	76	0.93	0.42
DEGME	120	0.91	0.48
TEGME	164	0.89	0.52
	Oligomers/Polymers		
isomaltotetraose	666	0.85	0.78
isomaltooctaose	1314	0.82	0.97
isomaltododecaose	1962	0.79	1.09
isomaltohexadekaose	2610	0.77	1.2
isomaltononadekaose	3096	0.76	1.27
PEO 200	200	0.89	0.61
PEO 300	300	0.87	0.66
PEO 600	600	0.84	0.75
PEO 900	900	0.81	0.88
PEO 1000	1000	0.81	0.91
PEO 1450	1450	0.78	0.91
PEO 4500	4500	0.69	
PEO 8K	8000	0.61	
PEO 14K	1.4×10^4	0.52	
PEO 100K	1×10^5	0.05	
PEO 600K	6×10^5	0	

[a] Abbreviations: MeOH, methanol; EO, ethylene oxide; DEO, di(ethylene oxide); TEO, tri(ethylene oxide); 2ME, 2-methoxyethanol; DEGME, di(ethylene glycol−methyl ether); TEGME, tri(ethylene glycol−monomethyl ether); PEO, poly(ethylene oxide).

data in ref 17 for lower molecular weight dextrans. For compounds with dimensions less than ~0.6 nm, it was not realistic to attempt hydrodynamic or scattering methods for determination of size. Therefore, molecular radii were obtained by computer modeling, as described in the Experimental Section, to give values of "R_{SAS}". This technique was also applied to the various oligomers with MW <2000.

The use of several techniques for assessing size raises questions about such comparisons. Unfortunately, R_S could not be measured for the oligosaccharides due to their low scattering intensities along with the modest quantity of sample available. However, several samples were in a size range for which radii could be obtained by varied methods. As shown in Table 2, R_η, R_S, and R_{SAS} could be obtained for PEO 1450 by viscometry, QELS, and computer modeling, and the results are identical within 0.1 nm. There is in fact no fundamental basis for expecting that these various measures be closely matched. Indeed, theoretical considerations show that R_S should be ~15% smaller than R_η for a flexible-chain polymer in a moderate-strength solvent,[22] which we observe for higher molecular weight PEO. Nevertheless, there is excellent agreement among these different measures of size for the low molecular weight compounds (with the exception of the datum for PEO 200, for which R_η was obtained by lengthy extrapolation). This result provides empirical support for the proposal that only modest differences exist among R_η, R_S and R_{SAS}. We therefore conclude that the marked change in slope at R = 0.6 nm in Figure 2 does not arise from the way in which R is measured but rather reflects the nature of the stationary phase.

Table 2. Radii Obtained by Various Procedures

	R_η (nm)	R_{SAS} (nm)	R_s (nm)
isomaltotetraose	0.7	0.78	
isomaltododecaose	0.9[7]	0.97	
isomaltododecaose	1.1[3]	1.09	
isomaltohexadekaose	1.3[2]	1.2	
isomaltononadekaose	1.4[4]	1.27	
PEO 200	0.4	0.61	
PEO 300	0.5	0.66	
PEO 600	0.7	0.75	
PEO 900	0.9	0.88	
PEO 1000	0.9[4]	0.91	
PEO 1450	1.1	1.04	1.0
PEO 4500	2		1.7
PEO 8K	2.9		2.1
PEO 14K	4		3.4
PEO 100K	11		12.2
PEO 600K	39		24.9

As shown in Table 1 and Figure 2, the elution volume varied among even the smallest solutes. Therefore, we could not arbitrarily select any one of these to obtain V_t. Indeed, the common use of D_2O or glucose for this purpose can be criticized since it implies zero molecular size. In considering the relationship between the elution of small molecules, such as D_2O or glucose, and that of oligomers or polymers, we note the abrupt change in slope at R = 0.6 nm. This suggests a discontinuity in the pore structure, which we may describe as arising from "micropores", which can accommodate only solutes smaller than 0.6 nm. The near linearity of the plot for larger R indicates that the larger pores may behave in a manner consistent with a cylindrical pore model. For such larger solutes, the proposed micropores are nonexistent. In other words, if we imagine a micropore-free packing, the data for all solutes would fall on the line shown in Figure 2, which would extrapolate to K_{SEC} = 1 at R = 0.

To determine the effective micropore-free pore volume, V'_p, we can simply compare the actual column, for which we have $K_{SEC} = (V_e - V_0)/V_p$, and a hypothetical micropore-free column, for which $K'_{SEC} = (V_e - V_0)/V'_p$. In the limit of R = 0, we find that $K_{SEC} = (0.90)^2$, from Figure 1, whereas according to eq 4 for the hypothetical micropore-free column, we should have $K'_{SEC} = (1.00)^2$. Thus, $K'_{SEC}/K_{SEC} = 1/(0.90)^2$. Consequently, we have $V'_p/V_p = (0.90)^2$, so that $V'_p = (0.81)13.78$ mL = 11.2 mL. The result is that micropores account for ~20% of the total pore volume.

Substitution of V'_p for $V_t - V_0$ in eq 2 enables us to calculate K'_{SEC}, which is presented vs R in the inset of Figure 2. The line connecting the data for solutes larger than 0.6 nm corresponds to the plot expected if the micropores vanished and D_2O were then used to obtain V_p. Aside from two of the oligodextran data, the remaining 18 points converge very well on a nearly linear curve extrapolating to K'_{SEC} = 0. Its weak curvature is entirely consistent with a continuous distribution of pores of similar (pseudocylindrical) geometry.[12] Marked deviations from this curve are observed for solutes smaller than 0.6 nm which have access to

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micropores. Permeation into these micropores clearly increases with decreasing solute size, so that the expected steric or "wall" effects persist even for the lowest molecular weights. Some deviations in this dependence are expected to arise from hydration effects, or possibly direct interaction with the stationary phase, but the data are remarkably uniform.

The congruence of the various dimensional measures observed here may be contrasted to the findings of Boyd et al.,[23] who investigated the permeation of oligomers into cylindrical cavities during SEC, using molecular dynamics simulations. Boyd et al. compared R_η, R_g (the radius of gyration obtained by computer modeling), and R_{ret} (the effective hard-sphere retention radius, i.e., the radius of a sphere that would yield the same K_{SEC}, according to eq 4 with $\lambda = 2$). For polystyrene, polyisobutylene, and polyethylene, in the range MW 100−700, significant differences were observed among R_η, R_g, and R_{ret}. At constant K_{SEC} (constant retention time), R_{ret} was typically 50−100% larger than R_g, while R_η was typically intermediate, but closer to R_g. Our limited results for PEO 1450, which show that R_{SAS} is slightly smaller than R_η, would suggest that R_{SAS} is intermediate between R_η and R_g.

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CONCLUSIONS

A rational definition of the pore volume, which enables one to relate the extent of macromolecular permeation to the equilibrium partition coefficient, K_{SEC}, should exclude microfissures that are accessible only to solvents or small solutes. The presence of such micropores is revealed by a discontinuity in the plot of the apparent K_{SEC} vs solute size R, in the case of Superose, at $R = 0.6$ nm. The corresponding plot for a hypothetical microfissure-free packing can be constructed by an adjustment in the pore volume so that the data obtained at $R > 0.6$ nm extrapolate to $K_{SEC} = 1$ in the limit of $R = 0$. This adjustment corresponds to an assignment of 20% of the pore volume to micropores.

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