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Thermodynamic equilibrium of the solute distribution in size-exclusion chromatography

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Abstract

Our understanding of the nature of solute retention in size-exclusion chromatography (SEC) is predicated upon an equilibrium, entropy-controlled, size-exclusion mechanism. The entropic nature of the separation depends, in turn, upon the solute distribution coefficient (K_{SEC}) being at (or close to) thermodynamic equilibrium. Classic experiments to confirm this assumption were performed over thirty years ago. Here, we combine information obtained from both flow and static mixing SEC experiments to show that the solute distribution in SEC is in thermodynamic equilibrium over a molar mass range extending one order of magnitude higher than previously measured (from 2×10^3 to 1.1×10^6 Da) using crosslinked polystyrene packing material of identical pore size (10^4 Å). The differences between our observations and previous ones conducted over three decades ago are ascribed, principally, to advances in stationary phase synthesis and column technology for SEC in particular and, secondarily, to improvements in the performance of the various instrumental components of liquid chromatographic systems in general.

Keywords: Retention mechanisms; Solute distribution coefficient; Static mixing; Thermodynamic equilibrium; Size-exclusion chromatography

1. Introduction

The retention mechanism in size-exclusion chromatography (SEC) is often referred to as an equilibrium, entropy-controlled, size-exclusion process [1]. Explaining SEC retention in thermodynamic terms is predicated upon the solute distribution being at (or close to) thermodynamic equilibrium. That said equilibrium exists in an SEC experiment is largely based on two independent types of study. The first type demonstrated that solute retention in SEC is independent of flow rate, i.e., that the separation is controlled mainly by the differential extent of permeation rather than by the differential rate of permeation. While initial experiments in this regard were performed over 30 years ago [2,3], additional confirmation of the flow-rate-independence has continued over the decades. The resurgence of interest in high-speed SEC, for high-throughput screening, etc. [4], has provided renewed experimental evidence of the flow-rate-independence using a variety of column packings, mobile phases, and analytes.

The second piece of evidence comes from so-called static mixing experiments. In these, a macromolecular solution of known volume and initial concentration (C_i) is mixed with a known amount of column packing material. After enough time for complete solute permeation to occur, the final solution concentration (C_0) is measured and compared to the initial concentration. If the solute distribution is in thermodynamic equilibrium, experimental values of the distribution coefficient (K_{SEC}) obtained via a flow SEC experiment should vary linearly with the parameter $(1-C_i/C_0)$ obtained from static mixing. The classic experiments in this regard were performed by Yau et al. over 35 years ago, examining the behavior of linear polystyrene (PS) with porous glass and crosslinked polystyrene (PS) packings [5]. In 1980, Janča et al. also examined the effects of concentration on SEC under equilibrium stationary conditions, using silica-gel column packing material [6].

Here, we revisit some of the static mixing experiments of Yau et al. using modern columns and packing material, prompted by a variety of related reasons. (1) A recent re-examination of classic stop–flow SEC experiments, used to study longitudinal diffusion in this technique, showed drastic differences between results obtained using modern

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columns and instrumentation versus data acquired using late-1960s technology [7]. (2) It appears extremely likely that the observed differences between modern and classic stop–flow experiments are due to the great advances in SEC column technology which have occurred over the last decades, in particular to the introduction of small particle size, rugged columns able to withstand elevated pressures as well as a variety of solvent/temperature conditions. Modern columns are virtually monodisperse with respect to both particle and pore size, and the particle surface is substantially "cleaner" (i.e., more inert) than the columns of yesteryear [8]. (3) SEC has advanced to the point where it can now discern not only amongst successive members of a homologous series, but also between conformational isomers and even between diastereomers. This power was recently focused on the study of O-linked disaccharides (and their monosaccharide constituents), where the K_{SEC} data were used to calculate solution conformational entropies and to quantitate differences in ΔS as a result of anomeric configuration, glycosidic linkage, or epimeric configuration [9]. As such, K_{SEC} is not only a fundamental descriptor of the size-exclusion separation process but also informs our knowledge of parameters vital to the study of molecular recognition processes, etc.

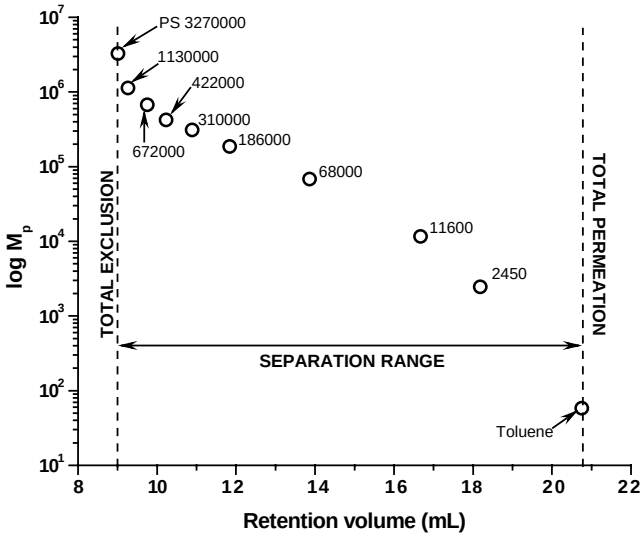

Fig. 1. Elution behavior of narrow polydispersity linear PS standards on 10 μm particle size, 10^4 Å pore size SEC columns. Toluene was used to measure to total permeation volume of the column set, PS 3,270,000 to measure the total exclusion volume. Each point represents the average of triplicate injections (M_p of each standard shown next to corresponding data point), with standard deviations substantially smaller than data points and, therefore, not shown.

2. Experimental

2.1. Materials

All PS samples were obtained from Polymer Laboratories (Amherst, MA, USA), except for PS 186,000 and 422,000, which were obtained from Toyo Soda (Tokyo, Japan). Molar mass polydispersities of all polystyrene standards were ≤1.07, molar mass values given here correspond to the peak-average molar mass (M_p); both values were determined by the manufacturers. Tetrahydrofuran (THF), toluene, and acetone were purchased from Fisher (Fair Lawn, NJ, USA). THF was spectrophotometric grade (UV cut-off ∼212 nm), inhibited with <0.025% butyrated hydroxytoluene (BHT) (λ_{max} ∼ 225 nm).

2.2. Flow size-exclusion chromatography: determination of K_{SEC}

Determination of the solute distribution coefficient (K_{SEC}) was performed using an SEC set-up consisting of a Waters 590 HPLC pump (Waters, Milford, MA, USA), a Waters 712 autosampler (Waters), and a Waters 410 differential refractive index detector (Waters). Separation was performed using two analytical SEC columns, 300 × 7.5 mm PL gel 10 μm particle size, 10^4 Å pore size (Polymer Laboratories) consisting of crosslinked polystyrene–divinylbenzene (PS–DVB). Column and detector temperatures were maintained at 35.0 ± 0.1 °C. Mobile phase was THF (degassed by He-sparging), at 1.0 ml/min, solution concentration was 1 mg/ml, injection volume was 100 μl. All results constitute averages of triplicate injections. The total exclusion (void) volume of the column set, V_0, was determined using PS 3270000, the total permeation volume, V_i, was determined using toluene, as shown in Fig. 1. All results were adjusted for minor flow rate fluctuations using the solvent/air peak common to all injections, as compared to the average value of this peak for triplicate injections of a solvent blank. Data collection was performed using Turbochrom Navigator, version 6.1.2.0.1:D19 (Perkin-Elmer, San Jose, CA, USA).

2.3. Static mixing experiments: determination of $(1 - C_i/C_0)$

For the static mixing experiments, 1 mg/ml solutions of the PS standards were prepared by diluting 14.0 mg of sample in 14.00 ml of THF (a THF blank, *sans* PS, was also prepared). Triplicate solutions of each standard were prepared. The solutions were shaken manually and allowed to solvate overnight. The next day, *even though the solutions were crystal clear*, 4.00 ml were removed and filtered through a 0.45 μm PTFE filter into a capped vial (the reason for filtration is given below). Two grams of porous stationary phase, 10 μm particle size, 10^4 Å pore size crosslinked PS–DVB from Polymer Laboratories (same material, particle and pore size as the SEC columns used to determine K_{SEC} above, and same manufacturer as well) was then added to the remaining 10 ml of solution (including to the THF blank). This mixture was shaken vigorously by hand and permeation of the solute into the pores was allowed to take place over the course of 24 h, after which 4.00 ml of solution was removed and filtered into a capped vial. An initial experiment showed

that after 24 h the mixture was cloudy due to a minuscule amount of suspended stationary phase and thus necessitated filtration. Because of this (and *not* because of incomplete dissolution or gelation), filtration of the first 4 ml removed (prior to the addition of stationary phase) was performed, in order that the two aliquots, before and after addition of stationary phase, experience the same preparation history (i.e., to avoid sample preparation bias).

Determination of C_i and C_0 was performed with a Cintra 40 UV-Vis dual-beam spectrophotometer (GBC, Hubbardston, MA, USA), using the absorbance at 262 nm. Absorption by PS solutions prior to addition of stationary phase was measured versus the absorption of the THF blank prior to addition of the stationary phase; the THF blank with added stationary phase was used as reference during the measurements of the PS solutions with added stationary phase. Data acquisition was performed using Spectacle, revision 1.70 (GBC).

3. Results and discussion

The classic static mixing experiments of Yau et al. measured the behavior of dilute solutions of PS in chloroform using, individually, 200 Å porous glass and 10^4 Å crosslinked PS [5]. In the porous glass study, K_{SEC} was calculated from experiments conducted at 1 ml/min flow rate, and the relationship between the solute distribution coefficient and $(1 - C_i/C_0)$ was perfectly linear between molar masses of 104 Da (styrene monomer) and 19800 Da, the separation range of the columns. The experiments using crosslinked PS packing material were conducted at two different flow rates differing by one order of magnitude, 1 and 10 ml/min. At 1 ml/min, the relationship between K_{SEC} and $(1 - C_i/C_0)$ was linear between 2030 Da (the lowest molar mass examined) and 119000 Da, but the behavior of PS of higher molar mass (247,000, 411,000, 860,000, and 1,800,000 Da) deviated from linearity. The fit between parameters was worse at the higher flow rate, with the high molar mass samples (PS 247,000 and above) deviating from linearity even more than their lower molar mass counterparts. The deviations from linearity in the experiments using crosslinked PS were attributed to lateral diffusion caused by velocity non-uniformity across the column cross-section [5,10,11].

As columns packed with crosslinked PS are widely used in organic SEC separations [7,9,12,13], and for the additional reasons mentioned in the Introduction, we decided to revisit these static mixing experiments using modern, state-of-the-art columns and packing materials. Our experiments were conducted with the same analyte (PS) over a virtually identical molar mass range (~2000–1,130,000 Da, see Fig. 1), the same packing material (crosslinked PS), the same pore size material (10^4 Å), and the same flow rate (1 ml/min). The behavior of linear PS in THF (used here) should be similar to that in chloroform (used by Yau et al.), based on the values of the Mark–Houwink exponent a (0.7–0.75 in both solvents at 25 °C [14]). The solution concentrations in the SEC experiments were likewise identical, as were the ratios of packing material to solution volume in the static mixing experiments. Our concentrations in the latter experiments were somewhat lower than those in the Yau et al. study (0.1% versus 0.5%), though this should have negligible influence on results. Instrumental differences are discussed later.

Table 1
V_R, K_{SEC}, and static mixing data for narrow PS standards

M_p (Da)[a]	V_R (ml)[b]	K_{SEC}[c]	$1 - C_i/C_0$[d]
2450	18.19	0.781	0.060
11600	16.67	0.652	0.115
68000	13.86	0.413	0.163
186000	11.84	0.241	0.217
310000	10.89	0.160	0.239
422000	10.23	0.104	0.250
672000	9.75	0.063	0.261
1130000	9.26	0.022	0.273
3270000	9.01	0.000	–

Total permeation volume (V_i = 20.76 ml) of column set determined using toluene.

[a] Peak-average molar mass values provided by manufacturer. $M_w/M_n \leq 1.07$ for all standards, as reported by manufacturer.

[b] Standard deviation (S.D.) ≤0.01 ml for V_R of all standards. Values were adjusted for minor flow rate fluctuations, as described in Section 2.

[c] Determined using Eqs. (1) and (2) from text.

[d] In all cases S.D. ≤0.003.

Values of the solute distribution coefficient are given in Table 1, along with the retention volumes (and peak-average molar masses) of the analytes in the flow SEC experiment and with the results of the static mixing experiment. Calculation of K_{SEC} was based on the retention volumes of the peak maxima (V_R), as well as on V_0 and V_i, as given by (1) [1]:

$$K_{SEC} = \frac{V_R - V_0}{V_p} \quad (1)$$

where

$$V_p = V_i - V_0 \quad (2)$$

V_p is the pore volume of the column set. V_0 and V_i correspond to the void volume and the total permeation volume of the column set, respectively, and their determination was described in Section 2.2. The measurement of C_i and C_0 was described in Section 2.3. While the 10^4 Å packing material is quoted by the manufacturer as possessing an upper separation limit of 600,000 Da (based on linear PS in THF at room temperature), Fig. 1 shows that we were clearly able to separate PS as high as 1,130,000 Da, a pleasant surprise. Fig. 1 also indicates that we could have performed more experiments using PS with $\sim 100 \leq M_p \leq 2000$ Da, though for the static mixing study we were limited in the number of experiments due to stationary phase availability (because of the high cost of this material) and it was the behavior of the high molar mass polymers that interested us most, as these were the species that showed large deviations from linearity in the experiments by Yau et al.

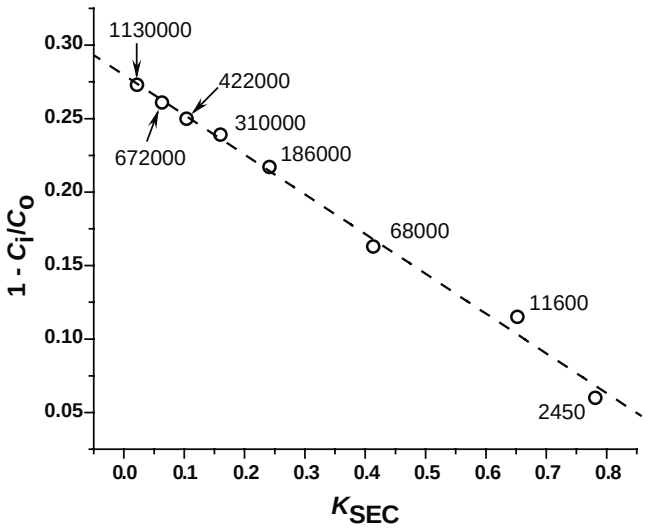

Fig. 2. Results of static mixing vs. flow SEC experiments, both using 10 μm particle size, 10^4 Å pore size crosslinked PS material from the same manufacturer. Values of K_{SEC} were determined from the data in Fig. 1 and Table 1 using Eq. (1) in text, as described in Section 2.2. Measurement of C_i and C_0 is described in Section 2.3. Each point represents the average of triplicate measurements, with standard deviations along both axes substantially smaller than data points and, therefore, not shown. Dotted line represents linear fit to the data ($r^2 = 0.994$). Numbers on graph represent M_p of each narrow polydispersity linear PS.

Fig. 2 shows the results of the static mixing experiments and their relation to K_{SEC}. Reproducibility was excellent, as evidenced by the fact that standard deviations along both axes were substantially smaller than the data points. It becomes immediately obvious that the relation between K_{SEC} and $(1-C_i/C_0)$ is linear over the entire range of separation, with the deviations from linearity previously observed by Yau et al. for high molar mass PS ($M_p > 120{,}000$ Da) absent in the present study. The values of the ordinate in Fig. 2 for each PS are virtually identical to the values measured by Yau et al. for PS of the same or very similar molar mass. In comparing abscissas we observe, for example, that in Fig. 2 of [5] K_{SEC} of PS 2030 is ~0.88 and K_{SEC} of PS 10300 is ~0.75, while in the present experiments (Table 1 and Fig. 2 of this manuscript) K_{SEC} of PS 2450 is 0.781 and K_{SEC} of PS 11600 is 0.652. This discrepancy, while small, may be due to the extended separation range of modern columns (in addition to the small difference between the molar masses of the analytes in the different studies). It could also be due to possible non-size-exclusion effects during the separation of styrene monomer used by Yau et al. to measure V_i. While styrene monomer and toluene (used to measure V_i in the present study) have very similar molar masses (104 Da and 92 Da, respectively), independent experiments have shown the former to elute at approximately the same retention volume as an *n*-butyl-terminated tristyrene oligomer, presumably due to enthalpic interactions between the double bond of the vinyl group of styrene and the column packing material [13].

While the exact reason(s) for the improvement in linearity of the K_{SEC} versus $(1-C_i/C_0)$ relation are not known, we consider it to be primarily related to the great advances in SEC column manufacturing over the last several decades [8]. Specifically, the uniformity of packing now achieved within columns, married to the uniformity in particle size and ruggedness of the packing material, would appear to reduce the lateral diffusion believed responsible for the behavior of the high molar mass species in the earlier experiments. (Also, the greater uniformity in pore size achieved in modern packings would help reduce stagnant mobile phase mass transfer). One must also consider, however, the advances that have also been made in all the instrumentation used in an SEC experiment. For example, Yau et al. used columns that were 4 ft long and injected 1 ml of sample solution into the system, while our columns were 300 mm × 7.5 mm each (two such columns were used) and our injection volume only 100 μl. Temperature control in the earlier experiments was difficult (not so nowadays), and instruments possessed siphon units that necessitated vapor feedback loops to eliminate errors caused by solvent evaporation from the siphon (none of this is necessary nowadays) [10].

Two other examples of how improvements in analytical column technology have changed our view of fundamental SEC principles are our stop–flow studies of longitudinal diffusion in SEC [7] and the work by Meehan and Oakley on combined SEC–HDC separation in a single system [15] (HDC: hydrodynamic chromatography). In the former, alluded to in Section 1, we were easily able to observe the effects of longitudinal (*not* lateral) diffusion for PS 2000 and other PS with $M_p < 30000$ Da after halting flow for <1000 min, while in experiments by Cooper et al. [16], published in 1969 using 2 ml injections into a 16 and a 50 ft column, no band broadening was observed for PS 2030 after 17 days! (Please note that the abscissa of Fig. 1A in [7] was incorrectly scaled by a constant −4.6 min offset; the correct retention time at the peak apex is not 11.5 min but is instead 16.1 min).

In discussing various theories of the separation mechanism in SEC, Yau et al. concluded in 1970 that "the velocity profile in the interstitial spaces does not provide the separation capability" [11]. Undoubtedly this statement was true at the time, as proved by the fact that styrene monomer (PS 104) and PS 860,000 eluted at virtually the same volume when using columns packed with smooth (i.e., non-porous) glass beads. Over two decades later, however, Meehan and Oakley showed how narrow polydispersity PS standards that were too big to fit into any of the pores of the column packing (i.e., polymers that should elute together at the total exclusion volume, regardless of differences in molar mass) could still be separated in the interstitial volume via a hydrodynamic chromatography mechanism (see Fig. 6 in [15]). Indeed, using a set of two 3 μm particle size SEC columns of mixed pore size in series, these authors separated PS in the range 162–30,300 Da by SEC and PS in the range 66,000–4,000,000 Da by HDC. It thus becomes obvi-

ous that as advances in both instrument hardware and column technology occur, not to mention in automated data collection and processing capabilities, the ability to observe both changes and improvements in retention, band broadening, and resolution needs to be capitalized upon.

4. Conclusion

We have described results of static mixing experiments which, combined with information obtained from flow size-exclusion chromatography studies, show that the solute distribution in SEC is in thermodynamic equilibrium over a molar mass range extending one order of magnitude higher than previously measured for crosslinked PS packing material of identical pore size. The differences between our observations and previous ones conducted over three decades ago are ascribed, principally, to advances in stationary phase synthesis ("cleaner" particle surfaces) and to column technology (greater uniformity of particle size, pore size, and packing) for SEC in particular [1,8] and, secondarily, to improvements in the performance of the various instrumental components of liquid chromatographic systems in general.

The improved behavior of current SEC columns has also been seen in recent work on longitudinal diffusion and on combined separation modes within a single column set where, in both cases, current measurements were in clear contradiction of earlier studies of decades ago.

The results described herein have bearing not only on our fundamental understanding of the retention mechanism in SEC but also on the thermodynamic information derived from this mode of chromatography. An example of the latter is the solution conformational entropy of monodisperse analytes, where the information obtained has the ability to inform our knowledge of structure–property relations related to molecular recognition and mimicry, docking and binding, etc.

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