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Longitudinal diffusion in size-exclusion chromatography: a stop-flow size-exclusion chromatography study

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Abstract

Band broadening in size-exclusion chromatography (SEC) has an adverse effect upon calculated molecular mass averages, distributions, and dilute solution data generated using single- and multi-detector systems. In the past, the longitudinal diffusion contribution to band broadening in SEC has been considered negligible. This assumption has been investigated by using a stop-flow methodology (SF-SEC) that maximizes the potential for longitudinal diffusion while minimizing that for mass transfer. Under the given experimental conditions, the effects of *B*-term band broadening were manifest only below 30 KDa, irrespective of chemical functionality or molecular mass polydispersity. This type of broadening was found to be flow rate-independent for a representative high molecular mass polymer.

Keywords: Longitudinal diffusion; Band broadening; Stop-flow size-exclusion chromatography

1. Introduction

> *''One cannot expect a molecule which follows a random migration path, full of frivolous excursions, to arrive after a fixed time at exactly the same point as its equally frivolous companions'', J. Calvin Giddings* [1]

According to the classic rate theory of chromatography, there are three main kinetic mechanisms responsible for band broadening in a packed column: (1) Eddy diffusion, (2) Longitudinal or axial diffusion, (3) Resistance to mass transfer. Accurate evaluation of the interdependence of these parameters leads to determining an optimum flow rate (u_{opt}) at which the height equivalent to a theoretical plate (*HETP* or, more commonly, *H*) is minimized. In traditional chromatographic techniques such as GC and HPLC, operating at u_{opt} allows for minimal band broadening and, thus, maximum chromatographic resolution to be achieved.

In a specialized technique such as size-exclusion chromatography (SEC), resolving multi-component mixtures is not usually an issue. The task in SEC is normally to effect separation of a polydisperse polymer in order to determine molecular mass averages and distributions, as well as dilute solution data that can be translated into morphological information about the polymer (e.g., intrinsic viscosity data into long-chain branching information). Band broadening in SEC can result not only in distortion of the calculated molecular mass distribution (*MMD*) of a polymer, but also in gross errors in molecular mass averages, specially in the number- and *z*-average molecular weights characteristic of the low (*Mn*)

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and high (*Mz)* ends of the *MMD.* Such errors will lead to concomitant effects on end-use properties. For example, processing characteristics of polymers such as brittleness and flow properties are normally correlated to *Mn*, while *Mz* is used to understand flex life and stiffness, and the weight-average molecular mass (*Mw*) is related to tensile strength and hardness, among others.

Peak variance in SEC is generally governed by a variety of mass transfer terms, while longitudinal diffusion is generally regarded as insignificant due to the slow diffusion of macromolecules in solution [2]. In this presentation we examine the latter assumption via a series of stop-flow size-exclusion chromatography (SF-SEC) experiments. The stop flow method has been used in the past to determine the apparent diffusion coefficients of analytes in SEC [3], as well as in traditional LC [4], and in CZE [5]. In this paper we use SF-SEC to examine longitudinal diffusion in dilute polymer solutions. The desire was to investigate whether *B*-term broadening is, indeed, negligible in SEC and, if not, to assess its dependence on the molecular mass, polydispersity, and chemical functionality of polymers. No attempt was made at determining effective diffusion coefficients (D_{eff}), as measurements were made for a single delay time, not for the series of delay times necessary for an accurate extrapolation to be made. Band broadening was evaluated using standard yardsticks such as peak height, peak widths, and reduced plate height. These results should have bearing not only on SEC data per se, but also on results from hyphenated techniques such as SEC–NMR, where analytes may be held in a stop-flow probe for as long as overnight, and on data from dynamic light scattering measurements, where researchers may trap analytes in the detector cell for long periods to increase the value of the intensity autocorrelation function. Additionally, accurate quantitation of the oligomeric species present in a polymeric sample has become extremely important from a legal standpoint, for the purposes of importing polymers into the United States and other countries [6].

2. Background

The traditional model of band broadening, as proposed by van Deemter et al. [7] recognized the three main kinetic mechanisms mentioned in the Introduction, and related them to plate height and flow rate via Eq. (1):

$$h = A + \left(\frac{B}{v}\right) + C_{\mathrm{m}}v + C_{\mathrm{s}}v \tag{1}$$

where *A* represents the contribution from ''eddy diffusion'' resulting from inhomogeneities of flow velocities and path lengths around packing particles, *B* the longitudinal or axial diffusion resulting from Brownian motion within the mobile phase and the tendency for Fickian molecular diffusion due to a concentration gradient, and the *C* terms are representative of the resistance to mass transfer at the solute to stationary phase interface. The subscripts ''m'' and ''s'' of the *C* terms refer to the mass transfer in the mobile and stationary phases, respectively. The plate height and flow rate are given in Eq. (1) as reduced variables, defined as:

$$h = H/d_{\mathrm{p}} \tag{2}$$

$$v = ud_{\mathrm{p}}/D_{\mathrm{m}} \tag{3}$$

with *H* being the plate height, *h* the reduced plate height, d_{p} the diameter of the column particle packing, *u* the linear velocity of the mobile phase, ν the reduced velocity, and D_{m} the diffusion coefficient in the mobile phase. The reduced variables were not present in the original van Deemter equation, being contributed rather the next decade by Giddings [1,8]. This approach tended to simplify the plate height equation considerably, as it scaled *H* to the particle size, and tied the kinetic processes to a balance between the convective velocity of the eluent along the column and the diffusive velocity of the analyte across a characteristic length (e.g., the particle diameter).

It soon became evident that the approach described by van Deemter et al. could not fully account for the observed plate height dependence on flow rate in a chromatographic column. Various methods have been proposed to correct this shortcoming, both by chemists [1,2,8–10] and by chemical engineers [11–14]. Whether the problem is approached from a molecular or a macroscopic level, however, the longitudinal diffusion term in the various plate height

expressions always remains inversely related to the velocity of solvent flow.

Over the decades a large number of contributions to the field of band broadening correction have been made. Virtually all of these can be traced to the original concept proposed by Tung [15], and a review of them is beyond the scope of this work. Particular to SEC is the method of Grushka involving exponentially modified Gaussian peaks [16], as it is currently employed in software packages specific to multi-detector size-exclusion chromatography [17].

The largest contributions to band broadening in size-exclusion chromatography have traditionally been considered to come from the mass transfer (C) terms of the van Deemter and related equations. In particular for SEC of macromolecules, stationary phase mass transfer (sometimes referred to as stagnant mobile phase mass transfer) appears to dominate. This process arises from the slow solute diffusion in and out of the pores of the packing particles. While some molecules are diffusing into the pores, others move farther downstream with the solvent. The longitudinal diffusion (B) term has generally been considered to be insignificant in SEC due to the slow diffusion of macromolecules in solution [2,18].

The stop-flow (SF) method in chromatography was reportedly conceived in 1962 during a taxi ride shared by Calvin Giddings and John Knox [8]. Besides advocating alternative means of transportation, this meeting yielded a method for studying the B term in band broadening. Variously referred to in the literature as stop-flow, interrupted flow, and arrested flow what is actually being done is to inject the sample onto the column and then allow it to travel part-way through the column before stopping the flow. After an extended period the flow is started again and the polymer is eluted from the column. As longitudinal diffusion is inversely associated with flow rate, a comparison of this ''stop flow'' chromatogram with a ''normal'' chromatogram (i.e., one obtained without stopping the flow) would serve to characterize the B term. This is done by comparing the variance of the stopped band versus the time of arrest. The slope of this plot yields the apparent or effective diffusion coefficient (D_{eff}). The ratio of this diffusion coefficient, obtained using a packed column, to that using an open tube gives the obstructive factor of the column.

One of the earliest and most quoted experiments using this method for SEC purposes was that of Cooper et al. [18] These researchers examined four polystyrenes ranging in molecular weights from 2030 to 160 000 Da. The delay times in the columns were as follows, with polydispersities (Mw/Mn) in parentheses: *PS* 2030 (2.22), 17 days; *PS* 10 300 (1.20), 90 min; *PS* 51 000 (1.42), 85 min; *PS* 160 000 (Mw/Mn not reported), 62 h. The conditions of analysis may best be characterized as ''industrial,'' not ''analytical:'' 2 ml injections into two columns, a 16 ft. column and a 50 ft. column. Results from these experiments showed a small but probably significant difference in molecular mass polydispersity for the smallest *PS* (M_{r} 2030) when comparing the normal and SF data, though no changes could be determined within experimental error for the other polystyrenes.

3. Experimental

3.1. Materials

Polystyrene and poly(methyl methacrylate) were purchased from Polymer Laboratories (Amherst, MA, USA). All other polymers were purchased from Scientific Polymer Products (Ontario, NY, USA), except for PVC, which was purchased from Pressure Chemical Company (Pittsburgh, PA, USA). Tetrahydrofuran was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Molecular weights for *PS* and PMMA correspond to peak-average molecular weights and for all other polymers to nominal molecular weights. Reported molecular mass data are those provided by the manufacturer.

3.2. Chromatography

For polymers with molecular weights below 100 KDa, 2 mg/ml solutions were prepared in THF. For polymers with $M_{\mathrm{r}} \geq 100$ KDa, 1 mg/ml solutions were prepared in the same solvent. Solutions were shaken vigorously on a laboratory shaker for two h, then allowed to solvate overnight. The following day, three 200 μl injections were performed sequentially using a Waters 712 WISP (Waters, Milford,

MA, USA), with run times of 30 min each. No filtration of the samples was performed. Separation occurred over two 5 μm Mixed C analytical columns (Polymer Labs), at 1.0 ml/min flow rate, with THF as mobile phase, and using a Hewlett Packard model 1037A differential refractometer as the concentration detector (Hewlett Packard, Palo Alto, CA, USA). Both column and detector temperatures were maintained at 35.0°C (+/− 0.1°C). Flow rate variations among these replicates were corrected using the three solvent/air peaks common to all injections. Immediately following these injections, 200 μl of a fresh aliquot of the same dissolution of sample was injected, and after 5 min the flow was brought abruptly (i.e., in a non-gradual fashion) to 0 ml/min and the polymer was allowed to remain on-column at zero flow rate overnight. The 5 min time-frame was chosen based on the fact that the largest polymer studied here, *PS* with $Mp = 1\,130\,000$ Da, had a retention time of ~11 min. Consequently, even the most highly excluded polymer in this study would experience a large portion of the pore volume available for separation. Additionally, the mixed bed nature of the columns assured that a statistically similar pore size distribution was being sampled in this way, which would not have been the case if two linear columns of different pore sizes had been connected in series. The next morning, after 960 min (16 h), the flow rate was raised abruptly to 1.0 ml/min and sample collection was begun. The entire procedure was repeated in triplicate for each sample.

Column performance was checked with the 400 KDa PMMA narrow standard. This sample was run in triplicate at both the beginning and the end of the experiments. All runs overlaid upon one another quite well after correcting for minor flow rate fluctuations (data not shown). This indicates that any degradation of the column packing that may have been caused by the sudden stopping and starting of solvent flow was minimal in nature.

3.3. Peak parameters

Data collection was performed using Turbochrom Navigator, version 6.1.2.0.1:D19 (Perkin-Elmer, San Jose, CA, USA). For graphical purposes, stop-flow and normal chromatograms were aligned visually. For analytical purposes, peak heights and widths were calculated from the refractometer's response as collected in Turbochrom.

4. Results and discussion

As described in the Experimental section, all polymers were allowed to remain on-column for approximately 1000 min. Measurements were made of peak height (P), of peak width at 50% peak height ($W_{0.5}$), and of peak width at 10% peak height ($W_{0.1}$). The ''5σ method,'' which measures the peak width at 4.4% peak height was not used, as it is generally considered to be too strongly influenced by peak tailing [19], which was expected to occur in the more polydisperse polymers.

Both qualitative and quantitative results are given in Table 1 for all polymers. Band broadening, as measured by the decrease in peak height and increase in band widths, was evidenced by this method. For the lowest molecular mass species, polystyrenes with molecular weights of 2000 and 5000 Da, measuring the change in peak height was sufficient to demonstrate band broadening. The difference between Fig. 1A in this paper and Fig. 1 of Ref. [18], where band broadening is barely observable for a polystyrene of equal molecular weight, is striking. The higher sensitivity to longitudinal diffusion of the present method is highlighted further by the fact that Cooper et al. held *PS* 2000 on-column for 17 days (24 480 min), while in these experiments hold time was less than 1 day (960 min).

Simple geometry indicates that peak height should taper off first with a decrease in broadening, followed by $W_{0.5}$, with $W_{0.1}$ being the most sensitive of the three parameters. This is the trend that was observed in these experiments. For quantitative purposes, and to allow comparison of results with data obtained on different column sets, the change in reduced peak height (Δh) was calculated from Eqs. (2), (4) and (5):

$$H = L/n \tag{4}$$

$$n = 5.55(t_R/W_{0.5})^2 \tag{5}$$

where L is the length of the column set, n the total

Table 1
Influence of molecular mass, polydispersity, and chemical functionality on longitudinal diffusion

Sample[a]	M_r (Da)	Mw/Mn	ΔP[b]	$\Delta W_{0.5}$[c]	$\Delta W_{0.1}$[d]	Δh[f]
PS	2000	1.05	++[e]	+++	+++	41 (2)
PS	5000	1.04	+	+++	+++	42 (2)
PMMA	7800	1.14	0	++	+++	22 (2)
PS	11 600	1.04	0	++	+++	33 (3)
PVF	10–15 000	5.62	0	++	++	25 (2)
PC	20–25000	2.74	0	+	+	14 (4)
PMMA	27 000	1.11	0	0	++	3 (2)
PVB	36 000	2.40	0	0	0	1
PVC	68 000	2.88	0	0	0	0
PBD	10 0000	2.18	0	0	0	0
PMMA	107 000	1.10	0	0	0	0
PS	170 000	1.04	0	0	0	0
PEMA	280 000	2.43	0	0	0	0
PMMA	400 000	1.14	0	0	0	0
PS	470 000	1.06	0	0	0	0
PS	1130 000	1.06	0	0	0	0

[a] Abbreviations: PS = polystyrene; PMMA = poly(methyl methacrylate); PVF = poly(vinyl formal); PC = polycarbonate; PVB = poly(vinyl butyral); PVC = poly(vinyl chloride); PBD = polybutadiene; PEMA = poly(ethyl methacrylate).
[b] Denotes decrease in peak height.
[c] Denotes increase in peak width at 50% peak height.
[d] Denotes increase in peak width at 10% peak height.
[e] 0 = Δ0–5%; + = Δ6–10%; ++ = Δ11–15%; +++ = Δ16–20%.
[f] Denotes percentage change in reduced peak height. Numbers in parentheses refer to standard deviations (std). Where no number is given, std < 1.

number of theoretical plates, and t_R the retention time of the analyte (measured at the peak apex).

As the molecular mass increases, broadening of the chromatographic bands is seen to decrease, as represented by the decreasing values of Δh. This is expected from the slower diffusion of the larger molecules in solution, but occurs irrespective of the chemical nature of the polymer. Represented in Table 1 are a fairly large variety of synthetic polymers. With certain exceptions, such as the longitudinal diffusion for *PS* 11 600 being greater than that of PMMA 7800, the tendency for a decrease in diffusion with an increase in molecular mass is evident from both the Δh data as well as from the trends in peak height and widths. It should be mentioned that both PVB and PVF are actually terpolymers. The particular PVB sample used in this study had a chemical composition of 88% butyral, 11% hydroxyl, and 1% acetate, while the PVF sample was 82% formal, 6% hydroxyl, and 12% acetate (percentages are on a weight basis).

The molecular mass polydispersities given in Table 1 are those provided by the manufacturer for *PS*, PMMA, and PVC. Values for the other polymers were determined in-house using THF as solvent/mobile phase and should be considered as relative to linear polystyrene. Polydispersities are seen to vary from essentially monodisperse ($Mw/Mn \leq 1.05$) for the *PS* standards, to polydisperse ($2 < Mw/Mn < 3$) for PC, PVB, PVC, PBD, PEMA, to highly polydisperse ($Mw/Mn > 5.5$) for PVF. What is observed is that, in addition to the chemical functionality independence of the longitudinal diffusion, the latter also decreases with increasing molecular mass regardless of the polydispersity of the polymer.

According to the data in Table 1, all evidence of longitudinal diffusion seems to disappear at molecular weights of approximately 30 000 Da, or somewhere between 27 000 and 36 000 Da to be exact, as measured by the yardsticks utilized here. Figs. 1 A–E and 2A give graphic evidence to these findings. Band broadening for *PS* 2000 and *PS* 5000 is rather severe, with changes in the reduced plate height on the order of 40%. For polycarbonate (PC), with molecular mass between 20 and 25 000 Da, no noticeable change in peak height is observed but

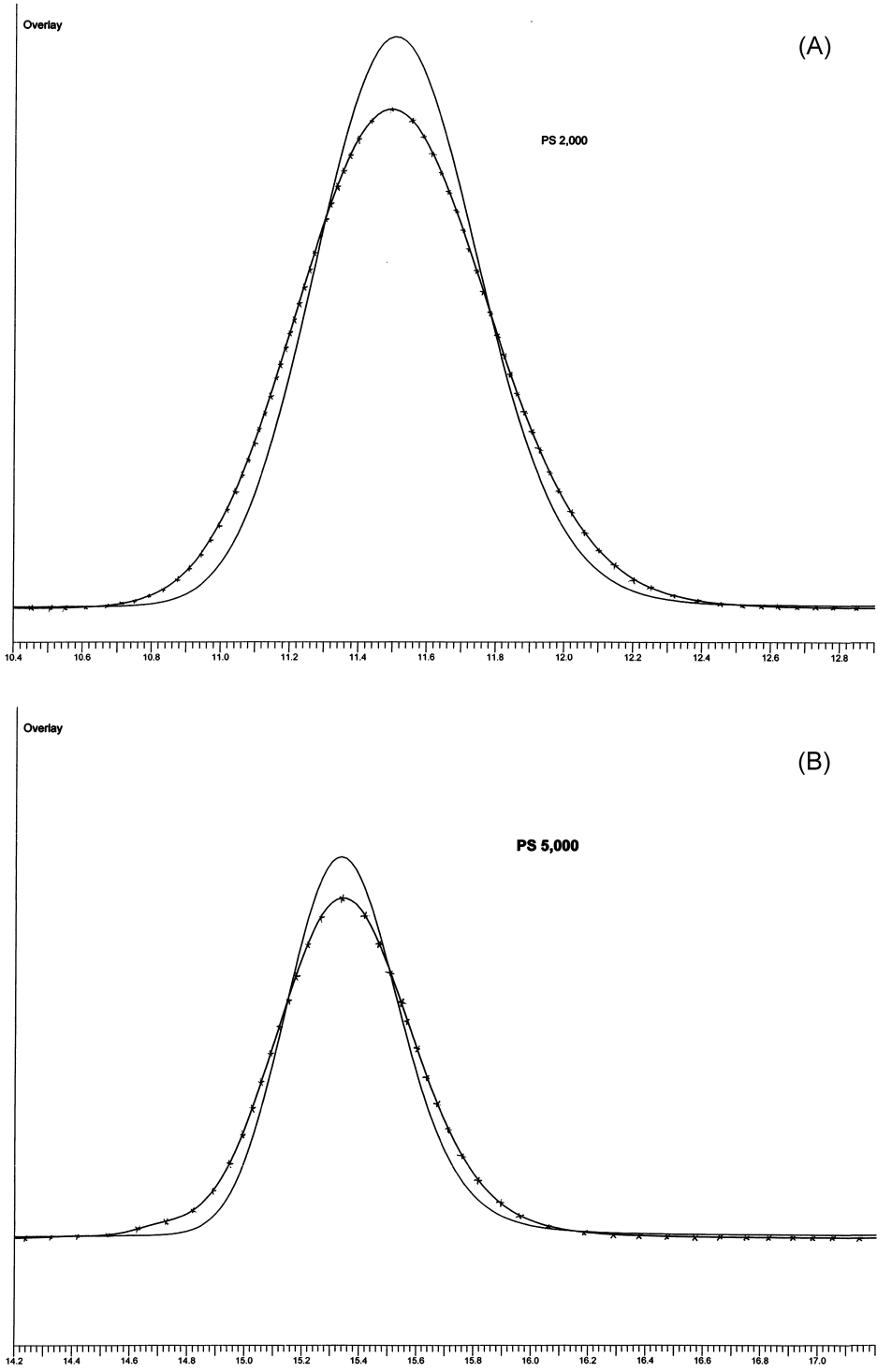

Fig. 1. Stop flow (xxxx) and normal flow (—) SEC chromatogram overlays. (A) *PS* 2000, (B) *PS* 5000, (C) PMMA 7800, (D) PC 20–25 000, (E) PVC 68 000. Abscissas on all graphs denote retention times of continuous flow chromatograms.

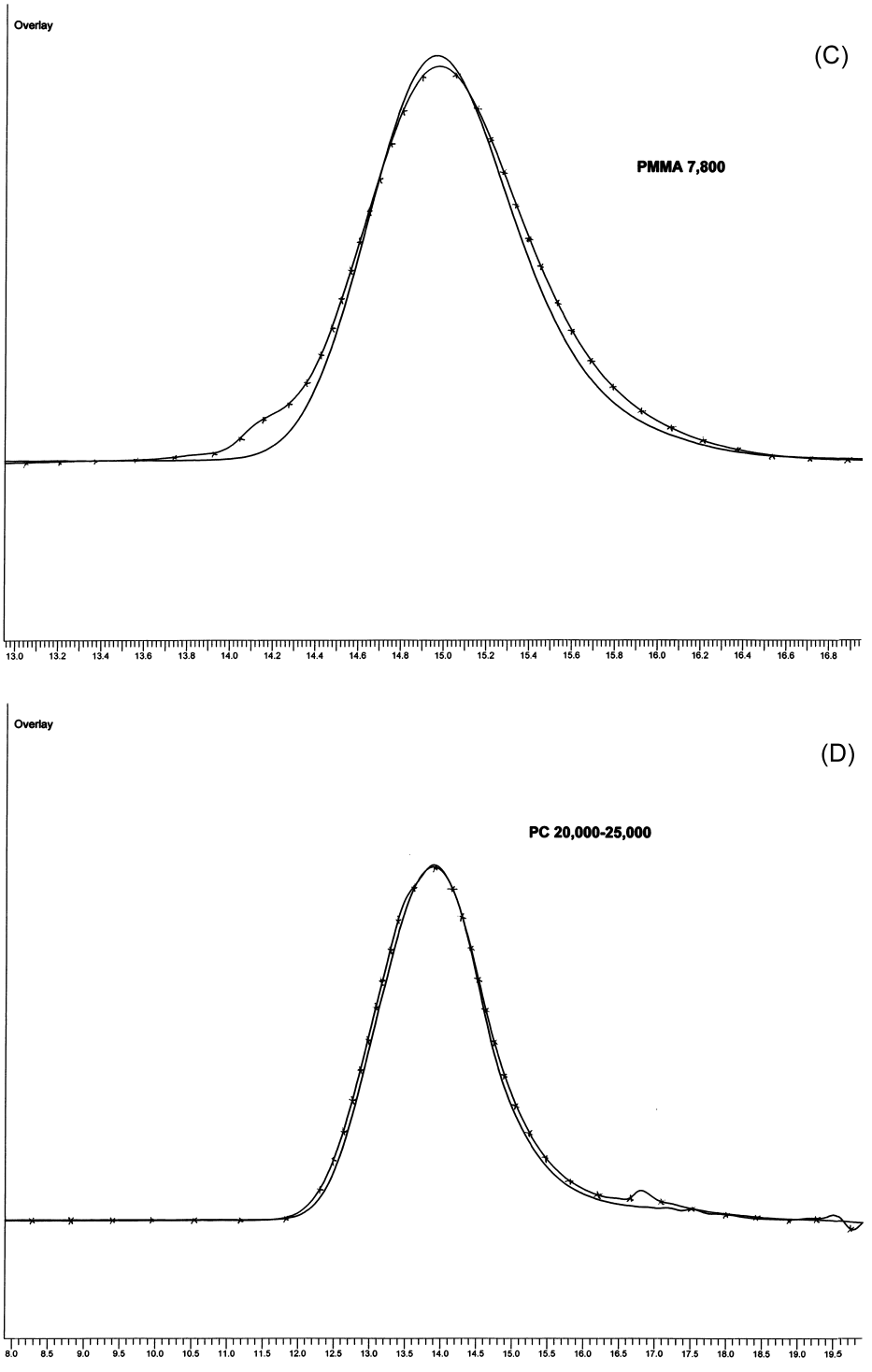

Fig. 1. (*continued*)

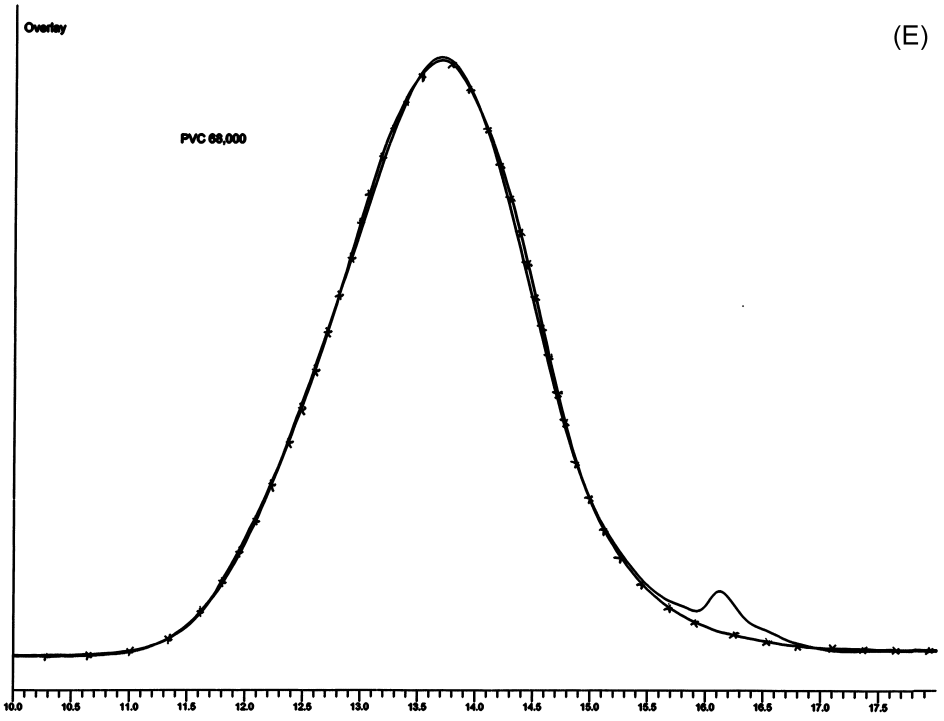

Fig. 1. (*continued*)

modest changes in band width at both 50 and 10% peak height (both at the 6–10% level) are still evident. These changes are reflected in the 14% change in h for PC. For PVC (M_r 68 000 Da) and PEMA (M_r 280 000 Da) the stop flow chromatograms are perfectly superimposable upon those obtained through normal (non-interrupted) elution. Not included in Table 1 are the results of an extended SF-SEC experiment involving the 400 KDa polystyrene narrow standard. This polymer was allowed to remain on-column over the course of one weekend, for 64 h (3840 min). The changes in peak height and peak width resulting from this extended stop flow time span were negligible, in agreement with SF observations by other researchers for high molecular mass polymers over comparable periods [18,20].

During review it was suggested that band broadening would manifest itself, even for the larger polymers, at a suitably low flow rate. To this effect, the PEMA sample was examined at 1.0, 0.5, and 0.1 ml/min. The results from this set of experiments are shown in Fig. 2A–C. The results at 0.5 ml/min are nearly indistinguishable from those at 1.0 ml/min, and both sets of data are quite similar to the results at 0.1 ml/min. It even appears that at 0.1 ml/min the stop-flow chromatogram displays slightly less band broadening at 10% peak height than the normal chromatogram. This is likely due to the fact that at this extremely low flow rate the refractometer signal was sometimes noisy did not always return to baseline. The failure to detect noticeable differences in band broadening as a function of flow rate is consistent with previous observations of the invariance of elution volume when flow rate was varied over two orders of magnitude, regardless of particle size [21]. Thus, the data presented in this paper support the long held view that separation in size-exclusion chromatography is controlled by the differential extent of permeation rather than by the differential rate of permeation [2]. These findings should have positive implications for those performing SEC of ultrahigh molecular mass ($M_r > 1 \times 10^6$ Da) polymers, where flow rates as low as 0.1 ml/min may be employed in order to avoid shear degradation of the macromolecular chains [22].

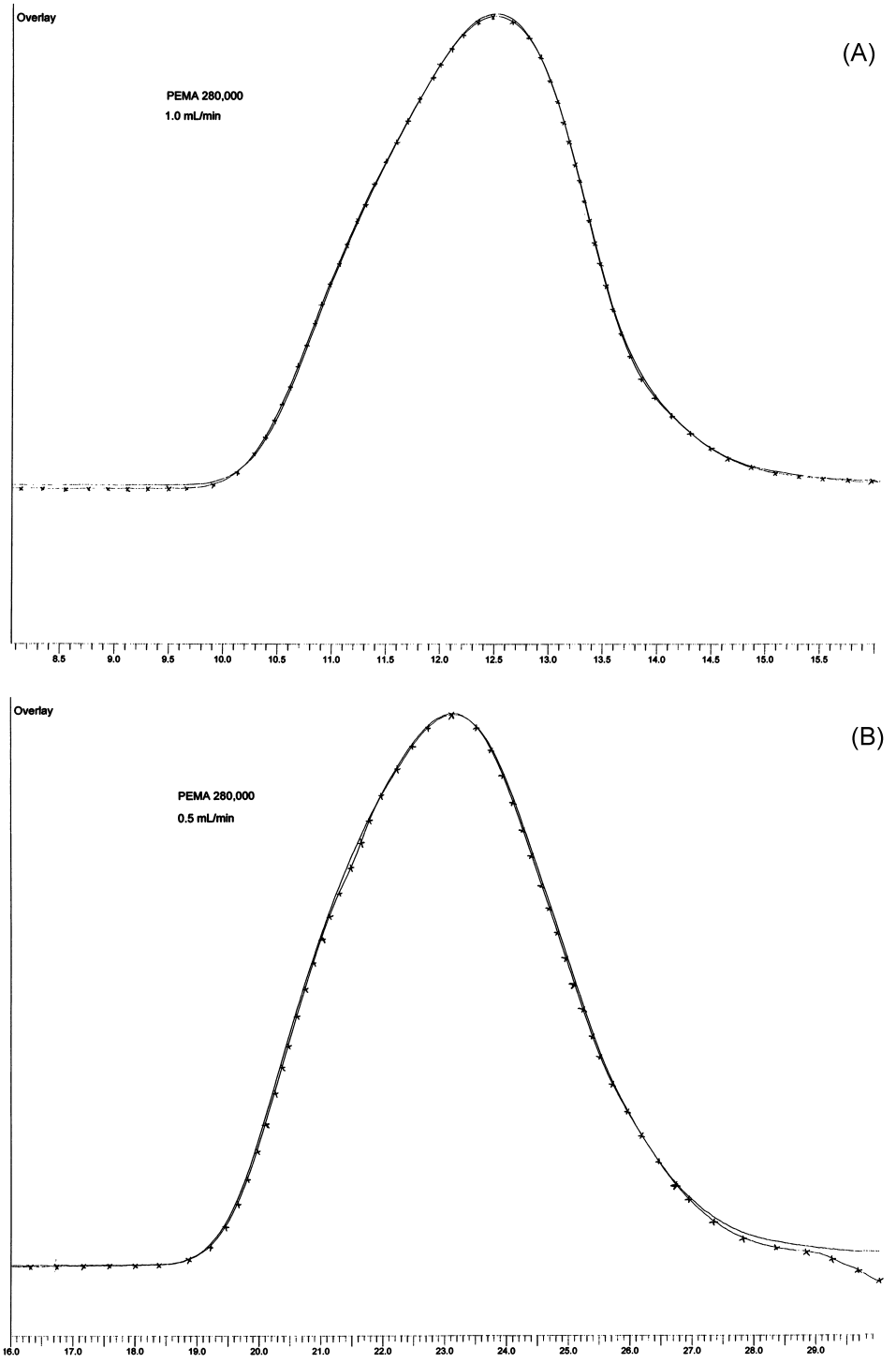

Fig. 2. Stop flow (xxxx) and normal flow (—) SEC chromatogram overlays for PEMA 280 000, as a function of flow rate: (A) 1.0 ml/min, (B) 0.5 ml/min, (C) 0.1 ml/min. Abscissas on all graphs denote retention times of continuous flow chromatograms.

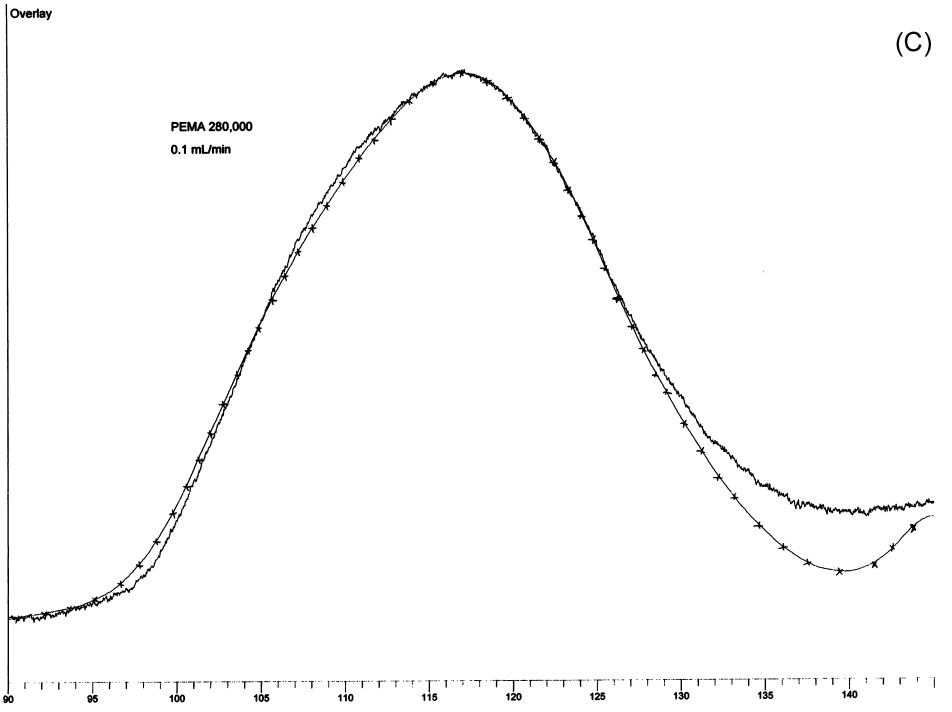

Fig. 2. (*continued*)

One additional factor that should be taken into account when comparing the stop flow results for two polymers, one small and one large, is the effect of the distribution coefficient, K_{SEC}. This parameter represents the ratio of the concentration of polymer in the stationary phase (i.e., inside the pores, or in the so-called ''stagnant mobile phase'') to that in the mobile phase (i.e., outside the pores). It is thus related to the extent of permeation alluded to above. Arresting elution for both polymers after the same amount of time gives a K_{SEC} higher for the smaller polymer, as more of its molecules will have diffused into the pores of the column packing as compared to the larger polymer during the same period. Diffusion in the interstitial medium should be easier than in the restricted space inside the pores. This would increase the statistical probability for longitudinal diffusion of the larger species, as more of the molecules in the injected sample would be located in the mobile phase outside the pores.

Evaluating the effects of longitudinal diffusion by examining the changes in peak asymmetry did not prove successful. This parameter is only truly useful when looking at narrow polydispersity polymers, as distinguishing the effects of polydispersity from those of asymmetry proves difficult. Nonetheless, even for the narrow standards no discernible trend could be found.

5. Conclusions

The effects of longitudinal diffusion on the band broadening of peaks in SEC were evaluated by the use of a stop flow methodology. These effects were found to be significant below ~30 000 Da, irrespective of the chemical nature or molecular mass polydispersity of the polymers. The lack of measurable longitudinal diffusion effects above this cutoff region occurs irrespective of flow rate, in agreement with classical theories of the retention mechanism in SEC. These results should prove helpful in attempting to correct measured SEC chromatograms for band broadening, in order to avoid over- and underestimation of molecular mass averages and distributions as well as of important dilute solution data.

They should also be useful to researchers in the relatively new arena of on-line SEC–NMR, many of which use stop-flow probes as part of their experimental set-up [23], as well as to those performing oligomeric SEC [24], as well as analysis of ultrahigh molecular mass polymers [22].

Not examined here were the effects of branching on longitudinal diffusion, as all the polymers studied were linear in nature. In particular long-chain branching, generally regarded as branches composed of 20 carbon atoms or more, may have an effect on the *B* term, specially when comparing a branched molecule to its linear counterpart of equal molecular mass [25–27]. This is due to the more compact shape of the former with respect to the latter, i.e., a branched molecule occupies a smaller hydrodynamic volume in solution than does a linear molecule. As branching is know to have a large effect on the draining properties of polymers in solution [28–31], it would not be surprising for it to affect longitudinal diffusion as well. Likewise unexamined were the effects of solvent and temperature.

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