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Size-exclusion chromatography—a review of calibration methodologies[☆]

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Abstract

Recent developments are reviewed in size exclusion chromatographic calibration methodologies, including direct calibration by using narrow and broad polymer standards and various instrumental methods (nuclear magnetic resonance, mass spectrometry, light scattering) as well as universal calibration with and without viscometry detectors, for simple and complex polymers.

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1. Introduction

Size exclusion chromatography (SEC) is a general name for the process of separation of molecules according to their size when a solution flows through a packed bed of porous packing. Within SEC, gel permeation chromatography (GPC) is the chromatographic separation of synthetic macromolecules with the use of porous gels or rigid inorganic packing particles, while gel filtration chromatography (GFC) or simply gel filtration is a similar process of separation for biological macromolecules (biopolymers).

In general, SEC is a liquid column chromatographic technique in which a sample solution is introduced into a column filled with porous packing and is carried through the column by solvent. Ideally, size separation is achieved by differential pore permeation. The volume of the pore, which is effectively accessible, is greater for a

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small molecule than for a large one. Therefore, larger molecules have shorter retention times in the pores of the packing than smaller ones and are eluted from the column earlier.

The first report of chromatographic separation of macromolecules according to size was made by Porath and Flodin [1] in 1959. They used cross-linked, semirigid polydextran gels devoid of ionic groups to examine various water-soluble polymers. Similar systems are still in wide use in GFC applications. In 1964, Moore [2] of Dow Chemical disclosed the effective use of cross-linked polystyrene beads for molecular size fractionation of synthetic polymers in organic solvents. Since molecular weight (MW) is frequently of more interest than molecular size, hence the need for MW calibration. It was evident that with proper calibration, SEC could reproducibly and accurately provide molecular weight distribution and molecular weight averages for linear homo- and uniform composition copolymers. These are often referred to as simple polymers in the context of SEC. With simple polymers and negligible band broadening, the SEC detector cell contains polymer chains having the same chemical composition and molecular weight. With complex polymers (polymer chains nonuniform in composition and/or polymers with a distribution of long-chain branching frequencies and branch lengths) and negligible band broadening, the contents of the SEC detector cell involve polymer chains with the same hydrodynamic volume but possibly with a wide range of compositions, molecular weights, and chain branching frequencies and lengths.

Over the four decades following those initial disclosures, much effort has been spent on the design and manufacture of a wide range of gels compatible with a variety of polymer and mobile phase types. In parallel, much has been done to improve the chromatographic equipment (solvent delivery systems, ancillaries capable of withstanding high pressures, and more powerful detectors). Early low-pressure SEC systems could yield accurate values of whole polymer MW and molecular weight distribution (MWD) in a matter of hours—a significant advance over classical techniques such as osmometry, light scattering, and ultracentrifugation. Nonetheless, the advent of microparticulate rigid packings, capable of withstanding high eluant flow rates and pressures, together with rapid online data acquisition and processing techniques, have culminated in the design of systems capable of yielding similar information within 10 min. There are, however, indications that when SEC analysis is performed on ultrahigh MW polymers, low flow rates are advisable because shear degradation might occur [3].

Today, SEC is the most widely accepted and used analytical method for the measurement of molecular weight distribution and molecular weight averages of biopolymers and synthetic polymers. However, because of many new developments in instrumentation, there are many different configurations of SEC systems used in different laboratories. Moreover, although on one hand there are many simple polymers in use or under development that are characterized by SEC, complex polymers that are soluble in the mobile phase are more often characterized by SEC in conjunction with online molecular weight detectors (e.g., light scattering and/or viscosity detectors). In the context of SEC, for a simple polymer, there is a unique relationship between radius of gyration (or hydrodynamic volume) and polymer chain molecular weight; hence—in effect—simple polymers are separated according to molecular weight. Complex polymers (polymers with long-chain branching or a distribution of composition, for example) on the other hand are

separated according to size; however, here, in the absence of peak broadening, the contents of the SEC detector cell will have the same radius of gyration but possibly a range of molecular weights. Online molecular weight detectors are essential for the quantitative SEC characterization of complex polymers.

Depending on the type of polymer (simple or complex) and instrumentation available, there are many methodologies that can be used for SEC calibration.

2. Developing SEC experimental methodology: specific and general considerations

There are several specific considerations one must make when developing a particular SEC method for the molecular weight characterization of a polymer of interest. These include choice of mobile phase, temperature, column packing type, column packing particle size, column packing range of pore size, detector type(s), molecular weight calibration method, and polymer concentration. In addition, to ensure high quality of the SEC analysis, there are also general considerations such as uniformity of the mobile phase flow rate during fractionation as well as a proper sample injection.

Since the focus of this survey is on SEC calibration for simple and complex polymers, calibration methods for various detector types and configurations will be discussed in detail, while other items will be briefly reviewed as relevant.

The simplest way to set up an SEC method is to utilize the existing instrumentation (e.g., HP 1090 or equivalent liquid chromatograph) and attempt to find a mobile phase/column packing combination that allows room-temperature polymer dissolution in a mobile phase compatible with the available column packing material. Failing to achieve satisfactory results, one has to resort to specialized SEC/HPLC instruments capable of working at elevated temperatures (e.g., Waters, and recently also Polymer Laboratories, are typical suppliers).

It should be stressed that a constant, reproducible solvent flow rate is a factor of extreme importance in accurate quantitative MW analysis with modern high-performance packing materials. When the total throughput time of the system may be as short as 10 min, even very small changes in flow rates can produce serious errors in MW determinations. Contemporary HPLC/SEC instruments utilize pumps that are able to produce high pressures (>1000 psig) to overcome the flow resistance due to the small gel particles packed in the columns. Special care has to be taken to ensure reliability of the pump, i.e., its ability to produce a constant flow rate over extended periods, by protecting against corrosion and plugging due to a possible presence of particles insoluble in mobile phase. It is common practice to insert a filter not only in the solvent feed line from the reservoir, but also a second one downstream from the pump, and to filter the sample solution before injection. Ideal injection should introduce a sharp plug of sample into the column with minimal peak spreading. Reproducibility of the amount of solution injected is an important consideration since, in some instances, the total amount of polymer injected needs to be known before one can proceed with MW calculations. It should be stressed that when dealing with ultrahigh molecular weight polymer and gel, improper filtration may remove a significant and important amount of the polymer sample.

3. Detectors in SEC

Various detectors can be used to continuously monitor the column effluent and to produce a signal which depends on the amount and/or MW of the polymer in the detector cell. Usually, a particular detector is performing measurements directly on a very dilute polymer solution. However, there are also evaporative detectors that remove the mobile phase by evaporation prior to detection.

3.1. Concentration detectors

Under ideal operating conditions, a concentration detector (CD) response is proportional to the total solute concentration in the detector cell. At least one concentration-sensitive detector has to be used with any SEC detector system if quantitative molecular weight determinations are to be achieved. Detectors that measure the differential refractive index (DRI) increment are the most commonly used as CD detectors. DRI detectors have the advantage of being able to respond to all solutes by proper solvent choice. Subject to solubility criteria, maximum sensitivity is obtained when solute and solvent differ in RI as much as possible. However, temperature instability can be a problem producing a baseline with a significant drift or even sinusoidal baselines if temperature changes are cyclical. It should be also pointed out that the DRI response factor, which is normally independent of molecular weight, might depend on molecular weight at low molecular weight levels as discussed below. Detectors that measure the ultraviolet (UV) and, less commonly, the infrared (IR) absorption of solutes are also in use as CD detectors. Nevertheless, their application in GPC is frequently hampered by the requirement that the polymer of interest has to contain a suitable chromophore and to be completely soluble in a mobile phase that is transparent to the incident light. UV photometers in particular are very sensitive, however, and in some cases, a few nanograms of solute is sufficient to give a strong signal. The UV instruments are also much less temperature-sensitive than DRI detectors. It should be noted that there are other possible concentration-sensitive detectors such as a density detector that utilizes the principle of the mechanical oscillator [4] or an evaporative light-scattering detector [5], but the former is seldom used and there are still problems in quantification of the signal for the latter. It is possible to use other types of evaporative detectors, e.g., as an interface to Fourier-transform infrared (FTIR) spectrometers [6,7]. There are also attempts to use detectors specific for a given group of polymers [8].

3.1.1. Detector response as a function of molecular weight

Detector response to the amount of polymer should be calibrated for different molecular weights over the range likely to be encountered under typical experimental conditions. The chromatograms obtained should be integrated and normalized peak areas plotted versus the molecular weight. Although usually it is assumed in SEC analysis that there is a constant response factor of the concentration detector, in actuality, this assumption is not valid below some threshold MW value for each polymer. All concentration-sensitive detectors are in a different way sensitive to monomer units in the polymer backbone and to chain ends that are chemically different from the main backbone units (e.g., in poly(eth-

ylene glycol), backbone units are $-\text{CH}_2\text{CH}_2\text{O}-$, whereas chain ends are $-\text{CH}_2\text{CH}_2\text{OH}$) and thus give a different detector response. Chain end effects are generally negligible for high MW polymers (since chain ends are “dilute” in such cases), but can cause considerable problems when one is examining oligomers. A recent study by Cook and Sible [9] has shown that in the case of differential refractometer, the response alters significantly for polystyrene in toluene when MW is below 1500 Da, whereas for poly(ethylene glycol) in chloroform, the DRI response increases with MW up to 20,000 Da. In other words, the refractive index increment (dn/dc) in this range is a function of MW. Similar observations were made earlier by Mori [10,11], who indicated that a solute and solvent pair having a smaller difference in refractive index has a larger MW dependency.

In the case of UV detector, the situation is more complicated since UV chromophores may be located in the repeat units or just the end groups, or both. Thus, if both repeat units and end groups contain same chromophore, the signal is proportional to the polymer weight concentration for the entire MW range. Interestingly, if only the end groups absorb UV radiation, the signal is proportional to the molar concentration of the polymer, i.e., the number of polymer molecules per unit volume. After determination of molar absorptivity, this measurement can be used to determine number average molecular weights. UV-chromophore containing groups can be introduced by derivatization of the end groups [12]. In the case of copolymers, UV molar absorptivity depends not only on the overall composition, but also on the sequence distribution [13].

3.2. Molecular-weight-sensitive detectors

These relatively new detectors are extremely helpful in SEC since the response of such a detector depends not only on the concentration, but also on the molecular weight of a polymer in the detector cell. There are the following major types of MW-sensitive detectors being used presently: low-angle laser light scattering (LALLS) and multiangle laser light scattering (MALLS) detectors and two types of viscosity (VISC) detectors (differential viscometers and single or dual capillary viscometers). In principle, also Fourier-transform nuclear magnetic resonance (NMR) and matrix-assisted laser-desorption/ionization mass spectroscopy (MALDI/MS) can be also used as SEC molecular-weight-sensitive detectors. Molecular-weight-sensitive detectors are essential to provide quantitative molecular weight of complex polymers by SEC.

4. Calibration for simple polymers

Different forms of SEC chromatograms can be obtained depending on the type of the detector signal used since different detectors can measure different properties of the eluate and/or the solute. A “classical” SEC chromatogram is presented in Fig. 1. In this chromatogram, the response of a concentration detector is plotted as a function of the retention volume, i.e., the plot depicts $F(V)$ versus V , where V is the retention volume.

Since SEC separates chemical species according to their size in solution, this plot reflects also a distribution of molecular sizes. Because the molecular size is related to the

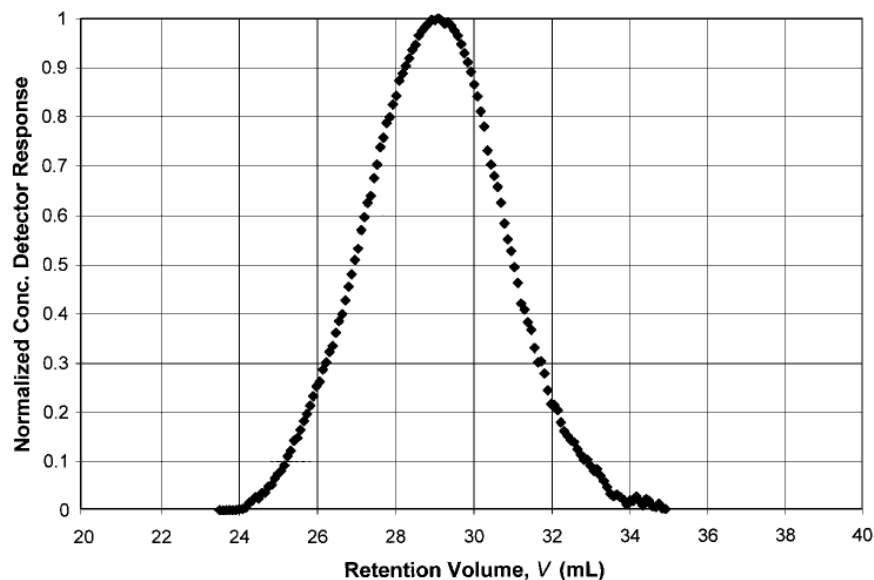


Fig. 1. Typical SEC chromatogram: the response of a concentration detector (CD) as a function of the retention volume.

molecular weight, SEC calibration is usually understood as the correlation of the molecular weight of a given polymer fraction with its elution volume (generally, calibration would be the relationship between elution volume and the property of interest). To determine this type of correlation, the analytical system has to be calibrated for the polymer of interest. Let us consider the total volume within the packed SEC column. This volume can be divided into three distinct components such that

$$V_c = V_d + V_o + V_p$$

where V_c is the total internal column volume, V_d is the “dead” volume occupied by the bulk of the packing material, V_p is the porous volume containing stationary solvent, and V_o is the interstitial volume occupied by moving eluant (also called the void volume). Separation in SEC is a partitioning of solute between mobile (interstitial) and stationary (in the pores) solvent. V_d takes no part in the chromatographic process; thus, separation in SEC has to be attained within a volume smaller than the internal column volume. Defining separation in terms of an SEC distribution coefficient K_{SEC} , we can write

$$V = V_o + K_{SEC}V_p$$

where V is the retention volume of a particular solute determined during an SEC experiment. For small molecules, which can access the entire porous volume for the column as well as the interstitial volume, $K_{SEC} = 1$; that is,

$$V = V_o + V_p$$

Solutes too large to fit in any of the pores can access only the interstitial volume; thus $K_{\text{SEC}} = 0$ and

$$V = V_o$$

This corresponds to the exclusion limit of the column and is the minimum value of the elution volume since no fraction of the sample can be eluted at the elution volume values lower than the interstitial volume. For solutes of intermediate size, there will be some fraction of the porous volume that is effectively accessible, depending on the size and shape (i.e., $0 < K_{\text{SEC}} < 1$). The relationship between K_{SEC} and the molecular weight of a macromolecule can only be determined accurately by calibration.

There have been many attempts to model K_{SEC} in terms of the size and shape of both solute and pore. The simplest model considers a system consisting of a spherical solute of radius R and a pore of radius a and length l . An excluded-volume effect prevents the center of the solute molecule from approaching the wall any closer than a distance R . This reduces the volume accessible to the solute to a smaller cylinder of radius $(a - R)$. In the smaller cylinder, the solute concentration is the same as in the interstices, while the shell of thickness R contains no solute. Hence, the average solute concentration in the pore as a whole is less than that outside. The fraction of the external concentration in the pore is given by the ratio of the two volumes, which leads to the following expression [14].

$$K_{\text{SEC}} = \Pi(a - R)^2 l / \Pi a^2 l = 1 - 2R/a + R^2/a^2$$

If the solute dimensions are much smaller than the pore, the squared term vanishes and we obtain

$$K_{\text{SEC}} = 1 - 2R/a$$

This discussion can be extended to random coils by imagining the coil domain as a sphere with r_g , the radius of gyration, taking the place of R . The foregoing equation, now expressed as an exponential series, becomes

$$K_{\text{SEC}} = \exp(-cr_g)$$

Since r_g is proportional to molecular weight M for a given polymer, we have

$$K_{\text{SEC}} = \exp(-kM)$$

Thus

$$V = V_o + V_p \exp(-kM)$$

Fig. 2 is semilogarithmic plot according to this equation where we have arbitrarily chosen $V_o = V_p = 20$ ml and $k = 10^{-5}$ mol/g. The plot extends from $M = 10^3$ to $M = 10^6$ Da and is clearly appreciably linear over the range 3×10^4 Da $< M < 2 \times 10^5$ Da.

It is commonplace in reality to find GPC systems for which, through judicious choice of column packings (i.e., as wide a range of packing porosities as possible to accommodate solutes of widely varying sizes), the calibration curve is approximately linear over four decades of M . The form of the V versus M calibration for the linear range thus reduces to

$$V = a - b \log M$$

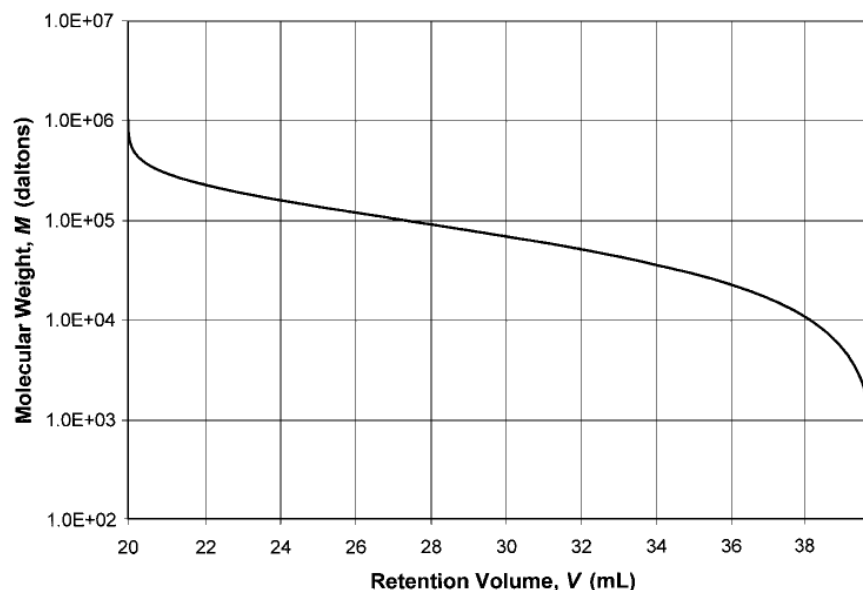


Fig. 2. Semilogarithmic plot of retention volume (V) versus molecular weight (M) according to equation $V = V_0 + V_p \exp(-kM)$ if $V_0 = V_p = 20$ ml and $k = 10^{-5}$.

where a and b are constants depending on instrumental parameters. The slope b defines the resolution or peak separation of the instrument. A shallow slope (low b) is favorable since there is a relatively large change in V for a relatively small change in M ; that is, two species of nearly equivalent M are better separated from each other when b is small.

4.1. Calibration with narrow MWD standards

The SEC apparatus is typically calibrated against linear narrow MWD polymer standards of known MW (from absolute methods such as light scattering or osmometry) and with a narrow molecular weight distribution ("narrow standards"). There are other methods based on broad standards, which are discussed below. When narrow standards are available for the polymer being analyzed, then calibration of a GPC instrument is straightforward: the standards are injected into the instrument to find their peak retention volumes and the calibration curve of $\log$ (molecular weight) against elution volume is constructed. It should be noted, however, when the chromatograms for the narrow MWD polymer standards are not symmetrical, the mean retention volume should be used. The asymmetry may be due to sample size overloading when the detector response factor is too low. This calibration curve can be used directly in determination of average molecular weights and molecular weight distributions for unknown samples of the same polymer. However, narrow standards for polymers other than polystyrene (PS) are still seldom available and narrow PS standards are employed to calibrate the system with the results for

the unknown polymer reported as “polystyrene equivalent molecular weights” [15]. Hence, the MW of many polymers is quoted relative to the MW of linear PS. For many relative comparisons, this is sufficient and PS standards are even used to evaluate MW distributions of biopolymers such as cellulose provided they are soluble in the same mobile phase [16,17]. Moreover, narrow PS standards are reported to be used to estimate true MWs for block copolymers if the mobile phase is a good solvent for both blocks [18]. On the other hand, standards of hydrophilic polymers, especially poly(ethylene oxide), have been also used for years to calibrate aqueous SEC systems [19–22], but in the case of biopolymers, they frequently have rather broad or even unspecified MW distributions. Recently, well-characterized narrow MW distribution standards of polysaccharides are becoming more available [23,24].

Typically, narrow standards are prepared either by preparative fractionation of linear polymers with broad MW distribution or by “living” anionic polymerization. It should be noted that recent developments in controlled (“living”) radical polymerization, which is much more versatile than anionic and can be conducted also in aqueous media [25], make it possible to prepare narrow standards for a broad spectrum of polymers and to extent the applicability of direct calibration towards many new polymers.

When the molecular weight for each fraction is determined, the number-average molecular weight ($\bar{M}_n$) and the weight-average molecular weight ($\bar{M}_w$) for a polymer composed of n fractions can be computed using the following equations with the assumption that the fractions are taken at equal retention volume intervals and that the concentration detector response factor is independent of molecular weight:

$$\bar{M}_n = \left(\sum H_i \right) / \left(\sum H_i / M_i \right)$$

$$\bar{M}_w = \left(\sum H_i M_i \right) / \left(\sum H_i \right)$$

where Σ means the summation over all fractions, i.e., from $i = 1$ to n , H_i is the height of the detector signal, measured from the baseline, for the retention volume V_i (H_i is a measure of polymer concentration in a polymer fraction), and M_i is the molecular weight of the polymer fraction at the retention volume V_i . These equations are valid when the detector response factor is independent of molecular weight. For DRI detector, this may not be true for a polymer sample whose $\bar{M}_n < 5000$ Da as it is discussed further.

4.2. Calibration with broad standards

There are several procedures when only broad MWD standards are available. However, a major drawback of such procedures is that unlike the peak position methods, instrumental peak broadening (which is discussed below) must be correctly accounted for if an accurate MW calibration curve is to be obtained. The “integral MWD” method requires the use of a standard for which the MWD is accurately known (which is seldom the case). The GPC calibration curve is obtained by matching those molecular weight and V values which correspond to the same value of sample weight fraction on the MWD and GPC elution curves separately.

The first of the so-called linear broad MWD standard calibration methods was developed by Balke et al. [26]. All that is required is a single broad standard of accurately known $\bar{M}_w$ and $\bar{M}_n$. A linear molecular weight calibration curve of the form

$$M(V) = D_1 \exp(-D_2 V)$$

where V represents the GPC retention volume, is assumed. Values of $\bar{M}_w$ and $\bar{M}_n$ are computed from the normalized experimental chromatogram $F(V)$ as follows

$$\bar{M}_w = \sum F(V_i) D_1 \exp(-D_2 V_i)$$

$$\bar{M}_n = 1 / \left(\sum F(V_i) / D_1 \exp(-D_2 V_i) \right)$$

A computer program iteratively adjusts D_1 and D_2 until agreement between calculated and known values is reached. Since peak spreading is not accounted for in this method, an “effective” rather than the true calibration is obtained. Improved versions of this procedure, which do account for peak spreading, have been proposed by Yau et al. [27]. This method can be extended to a nonlinear calibration curve by using more than one standard with independently determined molecular weight averages. Recently [28,29], SEC calibration using broad standards has been combined with the concept of universal calibration (which is discussed below) to determine the MWD for cross-linked globular proteins (haemoglobin and myoglobin “hyperpolymers”).

4.3. Direct calibration using nuclear magnetic resonance and mass spectrometry

Since the types of polymers available as calibration standards are still limited, another approach is to directly calibrate the SEC system using nuclear magnetic resonance (NMR) and mass spectrometry. Hatada et al. [30] and Ute et al. [31] have used powerful proton NMR instruments as SEC detectors to directly calibrate the SEC. They have demonstrated the potential of SEC-NMR combination by using anionically prepared polymethylmethacrylate (PMMA). This polymer has exactly one *t*-butyl group per chain and all monomer units are identical. Thus, the intensity of the proton NMR signal for the *t*-butyl group is a measure of the number of the chains, and the intensity of the proton NMR signal for the methoxy group is a measure of the number of monomer units. Calculation of the $\bar{M}_n$ for a polymer fraction is therefore straightforward (the ratio of normalized methoxy and *t*-butyl signals). If sufficiently narrow polymer fractions are taken, these $\bar{M}_n$ values can be used to construct a calibration curve for the polymer. Moreover, since the ^1H NMR signals for monomer units are proportional to the amount of the polymer in a given fraction, direct calculation of the MWD can be also attempted. Although the method has a great potential, there are some problems with axial dispersion since the NMR flow cell is relatively large (60 μl) compared to a typical SEC detector cell. The significance of axial dispersion in such a large detector cell has been demonstrated by using monodisperse PMMA fractions prepared by supercritical fluid fractionation [31]. One more disadvantage of direct coupling of SEC with ^1H NMR is the necessity to use deuterated solvents for SEC.

Another approach to directly calibrate the SEC is to use mass spectrometry to measure molecular weights of polymer fractions. There is a recent report [32] concerning online

coupling of SEC to Fourier transform mass spectrometry (FTMS) using a modified electrospray ionization (ESI) interface. Selected oligomer profiles for sodiated (1+ through 5+ charge states) oligomer ions of a narrow PMMA have been used to generate a calibration curve. MWDs have been calculated by using this calibration curve and the refractive index response. It should be noted that ESI can be used mostly for oligomers, as the spectra are quite complex due to the multiple charging. On the other hand, SEC/ESI/FTMS has allowed for end-group determination and characterization of a secondary distribution due to the formation of cyclic reaction products.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was once heralded as a method that would replace SEC in determining MWDs of simple polymers. However, it turned out that polydisperse polymers cannot be characterized reliably by MALDI-TOF-MS alone [33–36]. Nevertheless, a combination of SEC with MALDI-TOF-MS [37–39] can provide both direct calibration of the SEC system and end-group determination. Typically, analytical or semipreparative SEC is utilized to prepare narrow fractions that are measured for MW by MALDI-TOF-MS. The technique is rapid and requires a very small sample amount (nanograms). However, there is usually a significant effect of solvents used to prepare polymer, matrix, and cationization reagent solutions on MALDI-TOF-MS results, which requires optimization of the measurement in order to ensure reproducibility. Recently, microscale SEC has been coupled online with MALDI-TOF-MS using a robotic interface [33].

Alternatively, semipreparative HPLC can be used to obtain small amounts of monodisperse fractions of applicable polymers that, after MW determination with MALDI-TOF-MS, can be used for direct calibration of SEC [40].

4.4. Universal calibration

MW calibrations of the foregoing types suffer the considerable drawback of being valid for one particular polymer–solvent(mobile phase)–temperature combination. A calibration valid for a wide range of polymer and solvent types chromatographed on the same instrument would be very useful. In order to achieve true molecular weight information for a given polymer, an alternative is to use the universal calibration procedure. By “true molecular weight,” we mean values obtained by SEC that are equivalent to absolute values.

Universal calibration is based on the premise that in size exclusion chromatography, polymer molecules are separated according to their hydrodynamic volume (V_h), which is proportional to the product of molecular weight (M) and intrinsic viscosity ($[\eta]$). This can be shown as follows. The Flory–Fox equation [41] states that

$$[\eta] = \Phi \langle r_g^2 \rangle^{3/2} / M$$

where $[\eta]$ is the intrinsic viscosity of the polymer in the solvent of interest, Φ is a constant which has values ranging from $2.86 \times 10^{23} \text{ mol}^{-1}$ for a theta solvent to $1.7 \times 10^{23} \text{ mol}^{-1}$ for a good solvent, and $\langle r_g^2 \rangle$ is the mean-square radius of gyration for the polymer of molecular weight M . From this equation, we can easily see that

$$[\eta]M = \Phi \langle r_g^2 \rangle^{3/2}$$

Since the root-mean-square radius of gyration ($\langle r_g^2 \rangle^{1/2}$) is proportional to the root-mean-square end-to-end distance for a random coil, the product $[\eta]M$ is proportional to the volume occupied by a chain in solution. It was Grubisic et al. [42] who first proposed the use of $[\eta]M$ as the universal calibration parameter. Their original investigation, using THF as eluant for their SEC instrument, showed that a plot of $\log[\eta]M$ against V gave a common curve for the elution of solutes of widely varying molecular architecture [e.g., linear, “comb,” and “star” polystyrenes; linear poly(methyl methacrylates), polybutadienes, polyphenylsiloxanes, and various copolymers].

On the other hand, for each linear polymer, there is a Mark–Houwink equation relating molecular weight to intrinsic viscosity in the form

$$[\eta] = KM^\alpha$$

where K and α (alpha) are constants specific for the polymer–solvent system. Thus, if these Mark–Houwink constants are known both for the standards and for the investigated polymer, the true values of molecular weight for all polymer fractions can be calculated as follows. At any retention volume, hydrodynamic volumes of standard (subscript 1) and unknown (subscript 2) are equal according to

$$[\eta]_1 M_1 = [\eta]_2 M_2$$

Thus

$$K_1 M_1^{(1+a_1)} = K_2 M_2^{(1+a_2)}$$

Solving for M_2 gives

$$\log M_2 = [1/(1+a_2)] \log(K_1/K_2) + [(1+a_1)/(1+a_2)] \log M_1$$

Therefore, true MW values for unknown polymer fractions can be determined by using appropriate Mark–Houwink constants for both standard (K_1 and a_1) and unknown (K_2 and a_2).

Hamielec and Ouano [43] have generalized the universal calibration curve for complex polymers where now the curve is a plot of $[\eta]\bar{M}_n$ versus retention volume. Recently, Jackson et al. [44] repeated most of the original Benoit studies and tested again the concept of universal calibration by performing SEC on a series of linear polymers with chains of varying flexibility. They confirmed that despite widely different chain flexibilities, all polymers have a common $\log[\eta]M$ versus V plot. Cifra and Bleha [45] performed Monte Carlo simulations of partitioning in SEC in order to identify factors affecting the shape of the universal calibration curve. Using universal calibration, Lee et al. [46] determined individual MWDs of the components of binary polymer mixtures by SEC with multiple detection.

It has been found [47–49] that nonionic water soluble polymers such as poly(ethylene glycol) and most of the polysaccharides (with the exception of polysaccharides having rod-like molecules [49,50]) obey the SEC universal calibration if they are analyzed using a nonionic SEC system.

Moreover, during recent years, universal calibration procedures have been scrutinized by an IUPAC Working Party working on the determination of kinetic rate constants by using pulsed laser polymerization in conjunction with SEC. Since the SEC calibration is

the limitation to precise and accurate rate constant determinations, the IUPAC Working Party has concentrated on obtaining precise and accurate SEC results. As narrow standards for most of the polymers studied have been unavailable, validity of the SEC universal calibration has been carefully examined. Zammit et al. [51] have confirmed validity of the SEC universal calibration. However, it has been reiterated that the accuracy of SEC results strongly depends on the accuracy of the Mark–Houwink parameters. Thus, problems might occur in converting the hydrodynamic volume measured from universal calibration to molecular weight. When Zammit and Davis [52] have been comparing different calibration procedures, they have found that a single set of Mark–Houwink constants does not accurately represent the intrinsic viscosity–MW curve for MWs below 10 000 Da, thus confirming the findings reported in Ref. [53].

Another approach in utilizing universal calibration is to use a viscosity detector (VISC) in addition to the concentration-sensitive detector (CD).

4.4.1. Viscosity detectors

Incorporation of a viscosity detector (VISC) into GPC instrumentation requires the VISC to be used simultaneously with a CD. Viscosity detectors measure the pressure drop in a capillary, or a set of capillaries, to estimate specific viscosity of the solution. Single capillary viscometers [54] are usually strongly effected by any fluctuations in the flow of mobile phase and are typically used with a syringe pump to alleviate this problem. Alternatively, pulsation effects can be eliminated when two capillaries are used in series. The most sophisticated design is a differential viscometer that uses four capillaries that are arranged similar to a Wheatstone bridge [55]. In any of the mentioned viscosity detectors, the ratio of specific viscosity to the concentration can be a good approximation of intrinsic viscosity because polymer concentrations in SEC are very low. Thus, when concentration values are obtained from a concentration detector, measurements performed by viscosity detectors can be used to determine the intrinsic viscosity for each polymer fraction

$$[\eta](V) = [c(V)]^{-1} \ln[P(V)/P_0]$$

where $c(V)$ and $P(V)$ are, respectively, the concentration and pressure drop of solution eluting from the column at retention volume V and P_0 is the pressure drop of the pure solvent. The VISC detector is skewed to the high MW (low V) side owing to its greater sensitivity to high MW solutes. The value of $c(V)$ is determined from the concentration detector in the customary manner by use of equation

$$c(V)dV = mF(V)dV / \int_0^\infty F(V)dV$$

If the Mark–Houwink constants of the eluting polymer are known, the VISC becomes an absolute detector in the same sense as a light-scattering detector for simple polymers since

$$[\eta](V) = KM(V)^a$$

However, for a newly synthesized polymer, Mark–Houwink constants may not be known. In this case, a universal calibration curve is generated by using standards (e.g.,

polystyrene). Since VISC determines $[\eta]$, the universal calibration curve enables the determination of molecular weights for simple polymers for each value of V .

Thus, a combination of the two detectors (CD and VISC) enables one to overcome problems in converting the hydrodynamic volume values determined from the universal calibration curve to molecular weight values for a new polymer. However, this method of determination of molecular weights is also not problem-free. First, it is necessary to determine the exact amount of injected polymer and the exact values for peak integrals in order to calculate absolute polymer concentrations (for SEC with a CD only, relative concentration values are sufficient to obtain quantitative MW results). Second, an interdetector volume is difficult to determine precisely. Any errors in determining this volume can produce significant systematic errors in the measurement of polymer molecular weights and polydispersity. Originally, the interdetector volume was calculated from the volume of the detector cells and connecting tubing. However, it turned out that this value gave incorrect results and an “effective” interdetector volume (interdetector delay) had to be determined. Fortunately, this “effective” volume delay between the VISC and the CD can be determined easily by aligning the apexes of peaks obtained for injected monodisperse solutes. Thanks to preparative HPLC, such monodisperse standards with MW up to 1500 Da are becoming available for many polymers [56,57]. It should be stressed that this method is valid only for truly (molecularly) monodisperse samples; otherwise, a mismatch between the peak maxima from the two detectors exists. This is because the CD signal is proportional to concentration, but the VISC signal is proportional to the product of concentration and intrinsic viscosity. Therefore, the VISC signal reflects the product of concentration and molecular weight to the power of the exponent in the Mark–Houwink equation. This has been illustrated in Fig. 3 showing a simulated SEC-VISC signal corresponding to the SEC-CD trace presented in Fig. 1. Thus, for polydisperse samples, the peak apexes obtained from the two detectors are incomparable and other methods for the determination of interdetector volume have to be used. It has been recognized [58–60] that in the case of SEC-CD-VISC systems, five different types of calibration curves can be generated and used to determine interdetector volume. For example, one method is to use the intrinsic viscosity calibration curve obtained for a single broad MWD linear homopolymer and the corresponding intrinsic viscosity calibration curve obtained from narrow MWD standards, and then to find the value of interdetector volume that will allow to superimpose those curves [58–60].

It should be emphasized that universal calibration is valid only if the separation occurs according to the size exclusion mechanism. In other words, if there are significant polymer-stationary phase interactions (or nonsize exclusion effects), universal calibration is not applicable. Examples of nonsize exclusion effects include ion exchange, ion exclusion, ion inclusion, hydrogen bonding, or hydrophobic interactions. They may occur in SEC separations where at least one of the components (stationary phase, mobile phase, or polymers that are being separated) is highly polar or ionic [61]. The occurrence of nonsize exclusion effects can be discovered by utilizing viscometry and light scattering independently [62,63]. These effects can be controlled by changing the pH and/or ionic strength of mobile phase [64,65].

An option in determining polymer molecular weights without using Mark–Houwink constants is to combine the concentration-sensitive detector with a light scattering detector.

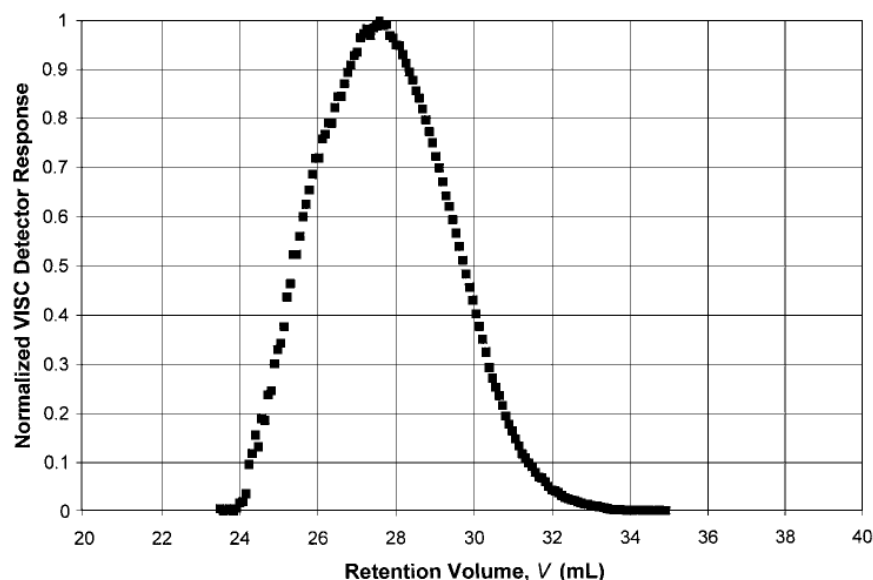


Fig. 3. Simulated SEC-VISC chromatogram corresponding to the SEC-CD chromatogram presented in Fig. 1.

In principle, this combination allows the determination of molecular weights without the use of universal calibration, although universal calibration curves, when applicable, might be still useful in “tuning-up” the system.

4.5. Calibration using light-scattering detectors

To determine molecular weights from light scattering (LS) experiments, we compare the intensity of light scattered at some angle θ or range of angles with that of the incident beam impinging on the scattering volume. The ratio of these intensities may be written as

$$R_{\theta} = I' r^2 / [I_{\theta}(1 + \cos^2 \theta)]$$

where R_{θ} is known as the Rayleigh ratio, I_{θ} and I' are, respectively, the intensity of incident light and of that scattered at angle θ , and r is the distance of the observer from the center of scattering.

In SEC applications, it is typical to pass column effluent through both a light-scattering (LS) detector and a CD detector. One obtains two chromatograms, one for a CD, as shown earlier in Fig. 1, and another for an LS detector as shown in Fig. 4. Notice the difference in peak profiles for the same polymer sample. The LS detector is more sensitive to the relatively few solute molecules of high molecular weight, which explains the apparent skewness towards the low-elution volume side.

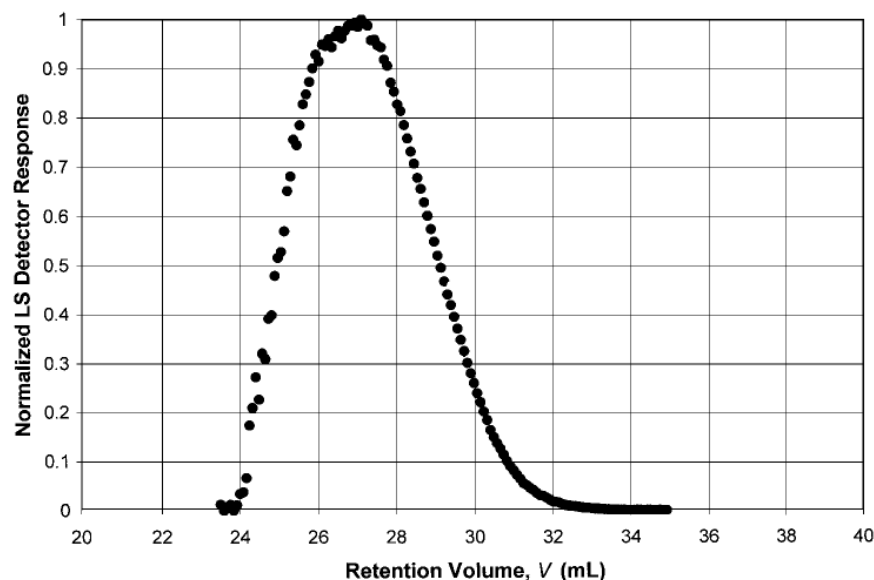


Fig. 4. Simulated SEC-LS chromatogram corresponding to the SEC-CD chromatogram presented in Fig. 1.

The LS detector gives directly the Rayleigh ratio as a function of V . The quantity used to calculate molecular weight at V , however, is the excess Rayleigh ratio $\bar{R}_\theta(V)$:

$$\bar{R}_\theta(V) = R_\theta(V)_{\text{sample}} - R_\theta(V)_{\text{solvent}}$$

The basic light scattering equation is

$$Kc(V)/\bar{R}_\theta(V) = [1/M(V) + 2A_2(V)c(V)][1 + (16\pi^2/3\lambda^2)\langle r_g^2 \rangle \sin^2(\theta/2)]$$

where $c(V)$ is the polymer concentration at V , K is an optical constant, $A_2(V)$ is the second virial coefficient, θ is the scattering angle, and λ is the wavelength of the incident light in the medium. The optical constant K varies from instrument to instrument, according to the light source (plane polarized or not) and instrumental geometry, but typically is given by

$$K = (2\pi^2 n^2 / \lambda_0^4 N_A) (dn/dc)^2 (1 + \cos^2 \theta)$$

where n is the refractive index of the solvent, λ_0 is the wavelength of the light in vacuo, and dn/dc is the refractive index increment.

A low-angle laser light scattering (LALLS) detector is frequently used and light scattered at very low forward angles (typically $\theta = 4-7^\circ$) is examined. In this case, extrapolation to zero angle customarily applied in classical “static” light-scattering experiments is considered to be unnecessary [66]. Then $M(V)$ is related to $\bar{R}_\theta(V)$ through

$$M(V) = [Kc(V)/\bar{R}_\theta(V) - 2A_2(V)c(V)]^{-1}$$

The values of $c(V)$ can be calculated from the CD response if the chromatogram is divided into volume increments of width dV . Then, from the injected mass of polymer m and the fraction $F(V)dV/\int_0^\infty F(V)dV$ of the total peak area represented by the increment, we have

$$c(V)dV = mF(V)dV / \int_0^\infty F(V)dV$$

The second virial coefficient (A_2) is a quantity that is a complex function of temperature, pressure, MW, size, and the thermodynamic parameters of the particular polymer–solvent system. The pertinent value of A_2 may be found in the literature; alternatively, it may have to be determined from a static MW analysis of the polymer in the solvent used for chromatography. In many cases, A_2 may be assumed to be independent of MW (hence V) without introducing significant error. However, when samples of wide MWD are considered, this is often not valid. From static analysis of narrow MWD fractions of the polymer of interest, we obtain the constants α and β in the equation

$$A_2(M) = \beta M^\alpha$$

where $A_2(M)$ may be used interchangeably with $A_2(V)$. The appropriate value of $A_2(V)$ is used in calculating $M(V)$. In reality, however, the magnitude of the contribution of $A_2(V)c(V)$ to $M(V)$ is relatively small, owing to the low polymer concentrations in GPC effluents. It becomes most important around the peak maximum, where $c(V)$ is at its highest. Once K , $c(V)$, and $A_2(V)$ are known, $M(V)$ values can be calculated. Thus, combination of both detector responses should lead to the absolute molecular weight at any chosen value of V across the entire chromatogram.

Using measurements at more than one angle, other types of light scattering detectors mentioned above can provide additional information leading to the estimate of the radius of gyration for the macromolecule [67]. If the second virial coefficient can be neglected, the radius of gyration can be determined from the slope of a plot depicting the equation

$$Kc(V)/\bar{R}_\theta(V) = [1/M(V)][1 + (16\pi^2/3\lambda^2)\langle r_g^2 \rangle \sin^2(\theta/2)]$$

It should be stressed, however, that for low molecular weight polymers, mean-square end-to-end distance is extremely small (in the range of 10^{-17} to 10^{-18} m²). Since mean-square radius of gyration $\langle r_g^2 \rangle$ is proportional to this value, for low molecular weight polymer species it may not be possible to measure $\langle r_g^2 \rangle$ accurately as the slope of the plot will have an extremely low value. Indeed, Jackson et al. [68] found that the lower limit for the determination of the root-mean-square radius by light scattering for monodisperse PS is ca. 10 nm (this corresponds to the MW of ca 70,000 Da). For a broad MWD polymer, the lower limit is even higher. It should be also emphasized that the scattering intensity depends on the optical constant that, in turn, depends, among others, on the refractive index increment (dn/dc). Thus, it should be remembered that polymers of varying composition would also have varying refractive index increments. In addition, any changes in mobile phase would also cause its refractive index and possibly also the refractive index increment to change. In any case, great care should be taken to ensure that proper refractive index increment values are used for calculations.

It should be pointed out that, similarly to the SEC-CD-VISC systems, the issue of interdetector volume delay is not a trivial one and can lead to major errors in MW determinations, although, in principle, the light scattering is an absolute method that should not require calibration. Again, an effective value of interdetector delay has to be determined, but, unlike the SEC-CD-VISC systems, monodisperse oligomers cannot be used reliably due to the sensitivity problems in light scattering in the range of low MW. It is thus necessary to rely on numerical methods to find the value of interdetector delay that would enable one to superimpose appropriate calibration curves [69,70]. Moreover, it has been found that with multidetector systems where detectors work in parallel (i.e., the column eluent is split), the interdetector delay varies with solution viscosity [52,71]. In this situation, universal calibration curves of hydrodynamic volume versus retention volume for different detectors are prepared in order to determine the “instantaneous” delay between the detectors [71]. Thus, the need to determine the interdetector delay in order to be able to match the responses of different detectors “slice by slice” leads actually to more calibration in these multidetector SEC systems than in the case of a classical SEC-CD. Inaccurate calibration in multidetector SEC systems can lead to systematic errors in MW determination due to inaccurate interdetector delay values. In the case of simple polymers, there are some strategies to eliminate systematic calibration errors of direct MW analysis when a multidetector system, which, in addition to the CD, includes also both light scattering and VISC detectors, is used. This can be done by (1) simultaneously using two independently calibrated MW determinations (SEC-CD-LS and SEC-CD-VISC) and comparing the results from each and/or (2) benchmarking the calibration by using narrow standards.

It is noteworthy that an SEC with both CD and light scattering detectors can be used to calibrate other SEC chromatographs. Using a tacit assumption of the validity of universal calibration, an SEC-CD-LS is used to establish a correlation between the molecular weight of the polymer of interest and the molecular weight of polystyrene (PS) at each retention volume [72]. After initial calibration with narrow PS standards, SEC chromatographs having only concentration detectors can rely on this relationship to determine the molecular weight of the polymer of interest.

Combining SEC with off-line light scattering can be used for a direct calibration of the SEC system [73].

5. Calibration for complex polymers

To reiterate, a complex polymer is a mixture of polymer chains that are not uniform in composition and/or a mixture of chains with distributions of molecular weight, long-chain branching frequency, and branch length. When band broadening is negligible, the detector cell will contain a mixture of polymer chains having the same hydrodynamic volume but with possibly wide ranges of chemical composition, molecular weights, long-chain branching frequencies, and branch lengths. The first question that must be resolved is that when employing the universal calibration curve, what molecular weight average, if any, should one use for the mixture of polymer chains in the detector cell at retention volume, V . SEC with online CD and viscosity detectors can provide a measure of intrinsic

viscosity versus retention volume. Hamielec and Ouano [43] have shown theoretically that the general form of universal calibration to be applicable to both simple and complex polymers must obey:

$$[\eta]_{\text{PS}}(V)M_{\text{PS}}(V) = [\eta](V)\bar{M}_n(V)$$

at retention volume, V , where $[\eta]_{\text{PS}}(V)$ is the intrinsic viscosity of the standard linear polystyrene of molecular weight $M_{\text{PS}}(V)$, $[\eta](V)$ is the intrinsic viscosity of the complex polymer mixture in the detector cell at retention volume, V , and $\bar{M}_n(V)$ is the number-average molecular weight of the complex polymer mixture at retention volume, V . Therefore, universal calibration with SEC (CD/viscosity detector) can provide a measure of $\bar{M}_n$ versus retention volume for the complex polymer sample. $\bar{M}_n$ for the whole polymer sample can be readily calculated, given $F(V)$ versus V obtained via the CD, as follows:

$$(\bar{M}_n)_{\text{whole polymer}} = 1 / \left(\int_0^\infty \frac{F(V)dV}{\bar{M}_n(V)} \right)$$

where $F(V)$ is properly normalized.

The intrinsic viscosity for the whole polymer sample is given by

$$([\eta])_{\text{whole polymer}} = \int_0^\infty [\eta](V)F(V)dV$$

SEC (CD/LS) can readily provide a measure of $\bar{M}_w(V)$ for the complex polymer of uniform composition in the detector cell, and the $\bar{M}_w$ for the whole polymer sample is then given by

$$(\bar{M}_w)_{\text{whole polymer}} = \int_0^\infty \bar{M}_w(V)F(V)dV$$

This is straightforward when the polymer chains in the detector cell have uniform composition and only one refractive index increment, dn/dc , is involved. When there is a distribution of compositions in the detector cell, the measurement of $\bar{M}_w(V)$ is unclear. When dealing with polymers having uniform composition and distributions at long-chain branching frequency and branch length, detectors which measure the intensity of scattered light as a function of angle (MALLS) can provide some useful information on radius of gyration of the complex polymer mixture in the detector cell. All of the polymer molecules in the detector cell (linear and branched) have the same radius of gyration and hydrodynamic volume. It should be pointed out that measures of $\bar{M}_n(V)$ and $\bar{M}_w(V)$ provide a measure of polydispersity, $\bar{M}_w(V)/\bar{M}_n(V)$, which if close enough to unity would suggest that the polymer chains in the detector cell at retention volume, V , are linear. This polydispersity is expected to increase above unity at lower retention volumes where the molecular weights are larger and the long-chain branching frequencies are higher.

SEC with online molecular weight detectors such as SEC (CD/viscosity detector) can provide most useful data for kinetic rate constant parameter estimation. For example, if a kinetic polymerization model can predict full molecular weight distributions, for polymer chains having zero, one, two, three, . . . long-chain branch points per polymer molecule, Zimm–Stockmayer long-chain branching equations [74] can be used to predict the

concentrations of polymer molecules having zero, one, two, three, . . . long-chain branch points and their molecular weights in the detector cell at retention volume, V . Thus, SEC (CD/LALLS/viscosity detector) measurement on complex polymers with uniform composition and distributions of long-chain branching frequency and branch length provides $[\eta](V)$, $\bar{M}_n(V)$, $\bar{M}_w(V)$, and weight fraction versus retention volume, V , data which is admirably suited for kinetic/reactor model parameter estimation.

Interestingly, in the case of complex polymers of uniform backbone composition but with short-chain branching (SCB), the hydrodynamic volume of the polymer backbone is not affected by the presence of the branches. Using a combination of temperature-rising elution fractionation (TREF) [75] with SEC, we have shown that for polyethylenes with SCB (branches up to six carbons in length), the apparent $\bar{M}_n$ determined from universal calibration does not change even though the frequency of the SCB increases dramatically [76]. This might pose a dilemma in calibrating SEC for such polymer systems as the detector cell, in the absence of band broadening, would contain polymers of the same backbone length but with different MW due to the presence of SCB. It has been shown [77] that Mark–Houwink plots for polymers with identical backbones but with different SCB frequencies show parallel lines. On the other hand, LCB should give Mark–Houwink plots with slopes different from those for linear polymers as it is evidenced in the case of polymer combs [78]. Thus, for some polymer by using “triple detector” SEC (CD/light scattering/viscosity detector), it should be possible to detect the presence of branching and distinguish between SCB and LCB [79]. Short-chain branching can be confirmed by using SEC in combination with FTIR [80].

It should be realized that the MWD curve of a polymer is important to understand rheological properties of the polymer. It turns out that using rheological properties of the polymer (e.g., complex viscosity), it is possible also to determine its MWD curve [77,81]. This MWD curve determined from rheological properties (referred to as “viscosity” MWD) may serve to verify the calibration of an SEC system. For linear polymers and polymers with SCB, there is an excellent agreement between the viscosity MWD and the SEC-determined MWD [77,81]. On the other hand, LCB causes great discrepancies between those two types of MWD curves. Thus, the degree of LCB could be identified by comparing the viscosity MWD curve with the SEC MWD curve [77,81].

6. Band broadening and consequences

6.1. Introduction

Band broadening, which includes instrumental peak spreading and skewing, has several consequences to the accuracy of the molecular weight information provided by SEC. Firstly, the detector response or chromatogram $F(V)$ may be distorted and broadened causing errors in the measured molecular weight distribution and molecular weight averages of the whole polymer sample. Secondly, the contents of the detector cell may be distorted. For example, the hydrodynamic volume or radius of gyration of all the polymer solute molecules in the detector cell at retention volume V may not be the same. The mathematical correction for band broadening to the detector response or chromato-

gram and the associated molecular weight distribution and averages for the whole polymer sample involve a solution of Tung's integral equation [82] for the corrected chromatogram, $W(V)$. However, the correction for band broadening in the detector cell at retention volume V requires a special solution of Tung's integral equation. This latter solution is particularly useful when employing online molecular weight detectors with SEC. Mathematical corrections for band broadening will be discussed later after the following detailed description of the band broadening are covered.

6.2. Instrumental peak spreading and skewing

6.2.1. Peak spreading

During an SEC experiment, a small volume of sample solution is injected as a very narrow band at the top of the column. As the band moves down the column, its width increases, partly because of size exclusion (smaller molecules being retained in the pores longer than larger ones), but also as a result of mechanical dispersion processes. These latter phenomena are responsible for the finite widths of peaks for monodisperse species, which should otherwise elute with the same very narrow bandwidth as the injected "slug." All such effects are detrimental to the resolution of the instrument; any amount of spreading interferes with the MWD information in the chromatogram. A small distortion in the shape of the chromatogram can cause significant errors in calculating MWs and MWDs. The chromatographer can minimize spreading but never completely eliminate it. Excessive spreading occurs, for example, if the gel bed is not homogeneously packed in the column, or if any flow channeling arises, necessitating careful packing procedures. In addition, the volume of all connecting tubing, detector cells, and any other extracolumnar elements should be minimized to reduce the possibility of solute mixing. Such features as solvent filters and pulse dampeners, for example, should be installed before the sample injection valve.

Eddy diffusion, in which diffusing solute molecules take different paths through the gel bed, is an important contributory factor to spreading. Another is mobile phase mass transfer, which arises because of the velocity profile of the solvent through the interstices between the packing granules. As in pure laminar flow, solvent velocity reaches a maximum at the center of the flow stream. A third process is stationary phase mass transfer, which arises from the time taken for solute to diffuse into and out of the pores in the gel. Large solutes with low diffusion coefficients are particularly badly affected by this phenomenon.

A polymer sample injected into an SEC instrument typically contains 10^3 – 10^5 individual species differing in MW by the monomer repeat unit. Owing to instrumental spreading, it is highly doubtful that resolution in SEC will ever reach the point of achieving baseline separation of the individual species. Typically, an SEC chromatogram is composed of a series of overlapping single-species detector responses.

Calibration of SEC instruments for spreading is not straightforward. Two "classical" methods of note are the reversed-flow [82] and recycle [83] techniques, both of which are rather demanding experimentally. In the former, more commonly used method, a sample is injected into the instrument in the normal way, but when the sample peak is halfway through the column, the flow is reversed. On the return to the top of the column, separation

due to size exclusion becomes completely canceled, but instrumental spreading effects continue. When the peak reaches the detector, now situated at the top of the column, its width is assumed to be entirely due to instrumental spreading. Tung and Runyon [82] performed reversed-flow experiments for a series of narrow MWD polystyrene standards to obtain a spreading calibration for their instrument, which they chose to formalize as a σ versus V function, where σ is the standard deviation of the flow-reversed Gaussian elution curve. The method does not work for skewed $G(V)$ since asymmetry in spreading does not cancel out upon flow reversal. Recently, Jackson [84] performed a computer simulation in order to evaluate the “effective volume shift” method for axial dispersion corrections in multidetector size exclusion chromatography. He found that although the method gave satisfactory results for SEC with an LS detector, it failed for SEC with an intrinsic viscosity detector, and he concluded that in this case, the conventional approach [27] outlined above gives more accurate results. Striegel [85] tried to evaluate the effect of axial diffusion on SEC band broadening by using a stop-flow technique. He found the effect significant only for polymers with the MW below 30,000 Da. The issue of band spreading continues to attract both theoretical and experimental studies [86–90].

6.2.2. Peak skewing

The ASTM Standard Method D3536 [91,92] includes the “Evaluation of Axial Dispersion” to check SEC operation for peak skewing. The appropriate equations follow:

$$\bar{M}_n(c) = \bar{M}_n(u)[X_1(1 + X_2)]$$

$$\bar{M}_w(c) = \bar{M}_w(u)/[X_1(1 - X_2)]$$

where

$$X_1 = 1/2[(\bar{M}_n(t)/\bar{M}_n(u)) + (\bar{M}_w(u)/\bar{M}_w(t))]$$

$$X_2 = (\Phi - 1)/(\Phi + 1)$$

$$\Phi = [\bar{M}_n(t)\bar{M}_w(t)]/[\bar{M}_n(u)\bar{M}_w(u)]$$

$\bar{M}_n(t)$ and $\bar{M}_w(t)$ are the true values measured by absolute measurement techniques such as osmometry and light scattering. According to ASTM Standard Method D3536, values of X_2 greater than 0.15 are unacceptable and modifications of mobile phase and/or column packing should be considered to reduce X_2 values to acceptable levels.

These equations have two different applications. The first is to test the SEC columns for packing imperfections (i.e., intracolumnar broadening) and for imperfections in ancillary equipment pieces associated with the chromatograph such as injection valve and detector (extracolumnar broadening). For this test, available narrow MWD standards with known $\bar{M}_n(t)$ and $\bar{M}_w(t)$ and soluble in the desired mobile phase are used. The molecular weight calibration curve is established using peak positions, and the detector responses for each standard are integrated to provide $\bar{M}_n(u)$ and $\bar{M}_w(u)$. The foregoing equations may then be

used to calculate X_1 and X_2 . Should X_2 be greater than 0.15 for a particular standard, but less than 0.15 for standards of lower molecular weight, it may be concluded that the mobile phase and column packing are satisfactory and that excessive skewing for the higher molecular weight standards is likely due to excessive polymer standard concentrations injected. At higher polymer concentrations, the molecular size (i.e., hydrodynamic volume and radius of gyration) is reduced causing a detector response tailing towards longer retention times and skewing. With narrow MWD polymer samples, size separation does not lead to as great a lowering of local concentration of polymer in the columns as with broader MWD samples for a given overall concentration. Skewing due to excessive polymer concentrations is therefore at a much lower level for polymer samples with a broad molecular weight distribution.

Although narrow MWD standards of any suitable polymer can be used to confirm that the SEC chromatographic equipment and particularly the columns and the quality of packing are acceptable, standards of the polymer of interest must be used to confirm that the concentrations of its samples injected do not lead to unacceptable levels of skewing. When accurate measurements of $\bar{M}_n(t)$ and $\bar{M}_w(t)$ by osmometry and light scattering are not available, SEC peak molecular weights for the prepared polymer standards using universal molecular weight calibration may be used (setting $\bar{M}_n(t) = \bar{M}_w(t) = M_{\text{peak}}$). It should be noted that a skewing factor of $X_2 = 0.1$ for an essentially monodisperse polymer would give an X_2 of less than 0.05 for a polydisperse sample with $\bar{M}_w/\bar{M}_n = 1.5$. This can be easily proven by taking an MWD with $\bar{M}_w/\bar{M}_n = 1.5$ and an MWD with $\bar{M}_w/\bar{M}_n = 1.0$ and comparing the concentrations at the peak position (the retention time at the peak position for both samples is the same).

Another cause of skewing or tailing of the detector response towards lower molecular weights is adsorption by various nonsize exclusion mechanisms of the polymer sample on the column packing. Whereas a reduction of polymer concentration injected in the absence of such adsorption on the column packing particles can eliminate skewing, skewing can never be eliminated in the presence of adsorption, and other means of minimization of skewing must be sought. Usually, one must resort to additives in the mobile phase, which preferentially adsorb on the column packing and reduce the adsorption of the polymer that is being analyzed. This can be risky and can lead to some errors, for example, the disturbance of a Donnan equilibrium for polyelectrolytes. Failing this, the final resort would be to change the column packing to one which does not present adsorption sites for the polymer of interest. Finally, the SEC methodology used is acceptable when skewing for broad MWD samples is less than 10% ($X_2 < 0.1$) and reproducibility of molecular weight averages is acceptable at levels $\bar{M}_n (\pm 5\%)$ and $\bar{M}_w (\pm 5\%)$ or better.

7. Concluding remarks

It should be emphasized that recent developments in size exclusion chromatography coincide with a great progress in synthesizing new tailor-made polymers and allow conducting in-depth studies of the newly synthesized polymers of ever-growing complexity. After proper calibration of all aspects of a multidetector SEC system, it is possible to obtain information about not only the molecular weight, but also structural character-

istics for polymers of various topologies such as combs, stars, and hyperbranched polymers [78,93–95]. Most interestingly, by using multiangle light scattering for direct calibration of SEC, it was recently possible to observe an unusual shape of calibration curves (molecular weight and/or radius of gyration versus retention volume) for some complex polymers [96–98]. Furthermore, by combining SEC with other fractionation/separation techniques, additional information about details of polymer architecture can be obtained [76,99,100].

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