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PERSPECTIVE ARTICLE

Size-Exclusion Chromatography: A Twenty-First Century Perspective

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Abstract
Now in its sixth decade, size-exclusion chromatography (SEC) remains the premier method by which to determine the molar mass averages and distributions of natural and synthetic macromolecules. Aided by its coupling to a variety and multiplicity of detectors, it has also shown its ability to characterize a host of other physicochemical properties, such as branching, chemical, and sequence length heterogeneity size distribution; chain rigidity; fractal dimension and its change as a function of molar mass; etc. SEC is also an integral part of most macromolecular two-dimensional separations, providing a second-dimension size-based technique for determining the molar mass of the components separated in the first dimension according to chemical composition, thus yielding the combined chemical composition and molar mass distributions of a sample. While the potential of SEC remains strong, our awareness of the pitfalls and challenges inherent to it and to its practice must also be ever-present. This Perspective aims to highlight some of the advantages and applications of SEC, to bring to the fore these caveats with regard to its practice, and to provide an outlook as to potential areas for expansion and growth.

Keywords Size-exclusion chromatography · History · Advantages · Applications · Caveats · Outlook

Introduction

As is, perhaps, the case with many of its users, size-exclusion chromatography (SEC), born in the 1950s, has gone from being the "hot, young, new thing" in the 1960s and early-70s, to irresponsible youth in the late-70s and 1980s (when at-best-dubious calibrant-relative results ran rampant through the literature), to questioning adult in the 1990s (when the combined applications on-line viscometry and light scattering gained increasing interest), and responsible (hopefully) parent in the early-aughts (during which we took stock of its limitations and grew to appreciate complementary techniques). Now, in this third decade of the twenty-first century, as SEC itself looks at its 60th birthday in the rearview mirror, it has begun to embrace its "senior statesperson" (read: grandparently) status among macromolecular separation techniques. With this comes rumination upon how life has been spent so far, on lessons learned, on what those next (though far from final) chapters may bring—and, of course, on how to spoil the grandchildren.

For those in need of a refresher, in SEC a dilute solution (or, in certain cases, a dilute suspension) of sample in a solvent is injected onto a column packed with porous, hopefully inert material. Assuming all sample components can enter the pores of the packing material (those too big to enter will simply elute, *en masse,* at the so-called "exclusion volume" of the column, in the absence of other separation mechanisms operating in tandem with SEC), the larger analytes, by virtue of their size, will sample a smaller effective pore volume than will their smaller-sized counterparts. The larger components in the sample will thus experience a shorter mean path through the column and will elute earlier than will the smaller sample components, because the latter spend more time within the pores exploring the larger effective pore volume available to them. Elution in SEC follows the well-known dictum "big ones come out first, small ones come out later," and we can think of SEC as an inverse molecular sieving technique. It is crucial to remember that elution in SEC is based on the solvated size of the analytes, not on their molar mass (after all, the technique is not called "molar-mass-exclusion chromatography"!). More fundamentally, elution is dictated by the solution conformational entropy of the analytes or, more accurately, by the entropic

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loss incurred when partitioning from the interstitial medium of the column into the pores of the column packing [1].

The main use of SEC remains the determination of macromolecule molar mass (M) averages and distributions. The true power of the method lies in its additional ability to inform our knowledge of other physicochemical properties of macromolecules, via couplings to a multiplicity and variety of detection methods, as well as by multi-dimensional couplings to other separation techniques. With power comes responsibility and practitioners must thus be aware of and admit the limitations of SEC and the pitfalls associated with its practice. It is the purpose of this Perspective to take stock of where SEC currently stands, as illustrated by a very few select applications from a laundry list of accomplishments; to point out some of the pitfalls and limitations; and to train our gaze into the future, asking ourselves where and how can the instrumentation and practice of SEC grow in the twenty-first century. First, though, let us look back upon how it all began…

Looking Back—Historical Background

The origin of SEC can be traced back to the classic work of Wheaton and Bauman of the Dow Chemical Company, in 1953 and, more specifically, to the 1959 work of Porath and Flodin from, respectively, the University of Uppsala and Pharmacia [2, 3]. In their paper, Wheaton and Bauman showed that non-ionic substances could be fractionated by employing ion-exchange columns, which indicated the possibility of analytes being separated from each other on the basis of their size in solution. It was, however, the research of Porath and Flodin that originally showed that this type of separation was possible. Employing columns packed with crosslinked polydextran gel swollen in aqueous media, the separation of various water-soluble macromolecules was demonstrated. They termed their method "gel filtration chromatography" or GFC and their work marked the advent of SEC as we today know it.

The development of other gels followed but, because these swelled only in aqueous media, their use was limited to the analysis of water-soluble substances. To overcome this limitation, John Moore of Dow Chemical used for the separation styrene/divinylbenzene gels crosslinked in a way that balanced rigidity with permeability [4]. Columns packed with these gels were connected to a differential refractometer (DRI) specially designed by James Waters of Waters Associates. This refractometer possessed a smaller cell than other commercially available refractometers of the time, had continuous flow in both the sample and reference sides of the cell, and could operate at temperatures of up 130 °C. Moore termed this incarnation of the technique "gel permeation chromatography" or GPC. It should be noted that GFC and GPC both separate by the same mechanism and are thus more aptly labelled, collectively and individually, as "size-exclusion chromatography;" maintaining a nomenclature difference based on the sole fact of whether one uses aqueous versus organic solvent for analysis is, at best, antiquated and, at worst, ludicrous.

The columns employed by Porath and Flodin, and by Moore, were all packed with semi-rigid networks of large particles with diameters in the approximate range of 75–150 μm. The columns could only be used at low flow rates and low operating pressures (< 250 psi or 1.7 MPa), which resulted in long, relatively inefficient analyses. The situation was remedied by the introduction of μ-Styragel, by Waters, in the 1970s. These columns were packed with smaller-diameter 10 μm particles, crosslinked to a degree that allowed them to withstand several thousand psi. This increased both chromatographic resolution and speed of analysis. Following this development, a variety of column packing materials have been introduced, ranging in size from around 3–20 μm and capable of separating anywhere from monomers and oligomers to ultra-high molar mass macromolecules and, even, sub-micron particles.

While both viscometry and static light scattering were employed as on-line SEC detectors in the 1970s, these generally consisted of home-built apparatus. Commercially available versions of these instruments did not become widely available until the 1980s. The combination of both these detectors, along with the perfunctory concentration-sensitive detector needed to help quantitate much of the information obtained from light scattering and viscometry, proved quite powerful in the study of macromolecular architecture, inter alia. This century has seen the increased use of so-called "chemical" detectors (e.g., infrared and nuclear magnetic resonance spectroscopes), able to inform our knowledge of the chemical composition of the eluting fractions, and of the combination of these detectors with their physical counterparts (static and dynamic light scattering, viscometry) [5]. The last of these is a powerful approach which can show how chemical changes across the molar mass distribution can translate into variations in the physical properties (e.g., chain rigidity) of copolymers.

On-line, comprehensive 2D-LC of macromolecules was first introduced in the mid-1990s [6]. This type of analysis, in which size-based techniques such as SEC often provide a critical separation dimension, can yield the combined molar mass distribution and chemical composition distribution (MMD × CCD) of copolymers [7].

Advantages and Select Applications

Perhaps the biggest advantages of SEC are its conceptual and experimental simplicities, especially in the case of single-detector SEC involving only a concentration-sensitive

detector such as a differential refractometer (DRI) or Ultraviolet/Visible spectrophotometer (UV/Vis). For example, the general concepts behind an SEC separation were explained in the second paragraph of this paper, concisely and without any equations. (The author has enjoyed explaining the way SEC separates analytes even to 1st and 2nd grade elementary school students, who have shown wonderful grasp—better than that of many adults—of what happens in the experiment). As we shall see in the next section, however, this simplicity can easily turn from advantage to pitfall.

An initial and immediately grasped advantage of SEC was its ability to provide the molar mass averages and distributions of macromolecules in a single experiment. As Ouano pointed out in the early-1970s, "With the introduction of [size-exclusion chromatography]… [molar mass] distribution data for polymers took a sudden turn from near nonexistence to ready availability" [8]. Prior to SEC, a multitude of consecutive fractionations were required, a lengthy process which yielded only a discontinuous picture of the molar mass distribution (MMD). The ability to provide this information is due to SEC separating on the basis of analyte size, independently (at least in theory) of molar mass or monomeric composition. The on-line coupling of SEC to viscometry allowed for the determination of absolute (i.e., calibrant-independent) molar mass information through the universal calibration approach; the on-line coupling to static light scattering dispensed with the need to construct any type of calibration curve, either absolute or relative. Each of these approaches comes, of course, with its own caveats; the reader is referred to reference [1] for a discussion of these.

Another advantage of SEC comes from the fact that one knows when an SEC experiment is over. Unlike other liquid chromatography (LC) techniques, such as small-molecule LC and most of those falling under the rubric of interaction polymer chromatography (IPC [9]), where the solute distribution coefficient K can adopt values ranging from 0 to ∞ and some analytes may take hours or even days to fully elute, the solute distribution coefficient in SEC, K_{SEC}, has values between 0 and 1, inclusive. In the absence of non-size-exclusion effects, it is simple to calculate when an SEC run is finished and when to start the next run. This also makes the technique amenable to 2D couplings (see, e.g., Chapter 14 of [1]).

While the above K_{SEC} limits translate into a limited peak capacity for SEC vis-à-vis its enthalpically dominated interaction counterparts, the ability to couple columns of different pore sizes gives SEC a tremendous dynamic range, being able to separate within a single experiment across orders of magnitude in molar mass (again, remembering that separation proceeds by size not, strictly, by molar mass), as shown in Fig. 1 [1]. In this example, which employed SEC with online multi-angle static light scattering (MALS), DRI, and UV/Visible detection, not only could the MMD and associated statistical averages be determined but also the chemical heterogeneity of the copolymer, i.e., the change in monomeric ratio across the MMD, given here in terms of the change in the percentage of *N,N*-dimethylacrylamide comonomer.

Using individual pore size columns, members of various homologous series of oligosaccharides have been separated from each other (see, e.g., [11–14]), as have been macromolecules as large as 300 nm and, with great care, even as large as 500 nm in radius [15]. Recently, even the ideal (i.e., strictly entropy-controlled) SEC separation of a pair of diastereomers was achieved under typical conditions of analysis (see Fig. 2), without enthalpic contributions to the separation as demonstrated by its temperature-independence [16].

Most SEC experiments are conducted isocratically using a single solvent, to avoid the problems created by preferential analyte solvation when using mixed solvents [17]. This allows the accurate coupling of a variety and multiplicity of differential detectors (e.g., DRI, UV–Vis, viscometry, static and dynamic light scattering, etc.) which, as already mentioned, gives SEC tremendous characterization power.

Typical (and some atypical) applications of SEC include:

- Determination of molar mass averages and distributions.
- Determination of long- and short-chain branching (branch number and frequency, etc.) across the MMD.
- Determination of various size distributions and of size versus molar mass relationship.

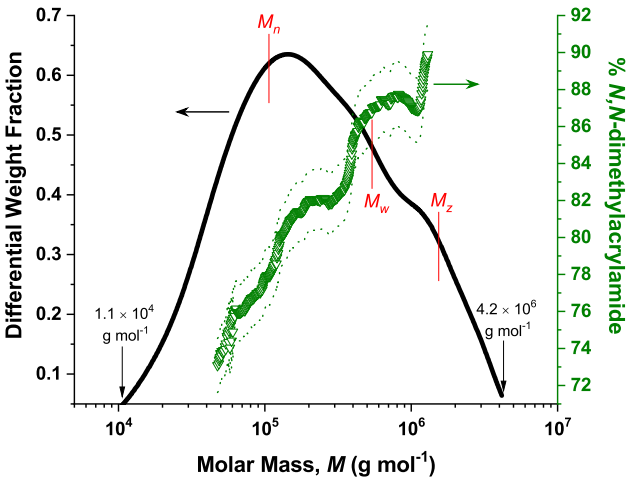

Fig. 1 SEC analysis of a copolymer covering over two orders of magnitude in molar mass. Molar mass distribution, associated averages, and chemical heterogeneity (given as percentage *N,N*-dimethylacrylamide comonomer; green inverted triangles; dotted lines denote ± 1 standard deviation) of a poly(acrylamide-*co*-*N,N*-dimethylacrylamide) copolymer, as determined by SEC/MALS/DRI/UV. $M_n = (1.06 \pm 0.08) \times 10^5$ g mol^{-1}, $M_w = (5.33 \pm 0.85) \times 10^5$ g mol^{-1}, $M_z = (1.52 \pm 0.17) \times 10^6$ g mol^{-1}. Columns: Four-column bank of PL Aquagel-OH 60, 50, 40, and 30 connected in series. See [10] for additional experimental details. (Adapted from [10])

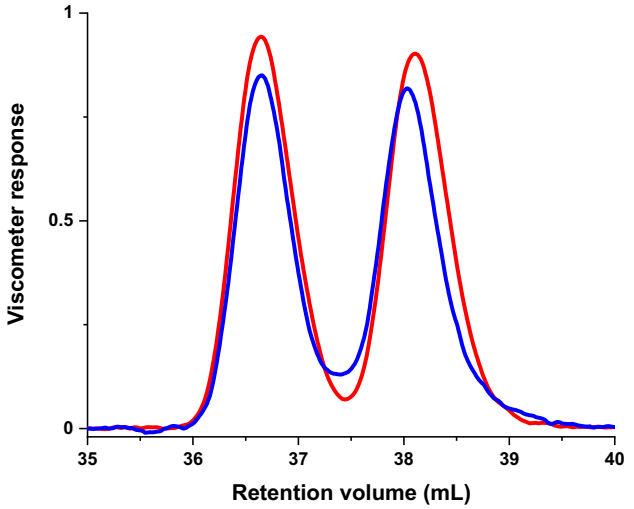

Fig. 2 Elution by an ideal size-exclusion mechanism. Overlay of SEC/viscometry traces of a 1:1 mix of the monosaccharides methyl-*α*-*D*-galactopyranoside (earlier-eluting peak) and methyl-*α*-*D*-mannopyranoside (later-eluting peak). Red: 25 °C; blue: 50 °C. All other experimental conditions equal in both cases. Percent change in K_{SEC} as a function of temperature was 1.03 for the mannopyranoside, 0.423 for the galactopyranoside, indicating an absence of enthalpic contributions to the separation. Columns: Bank of four 120 Å nominal pore size, 7.8 mm × 300 mm Ultrahydrogel columns. See [16] for additional experimental details (Adapted from [16])

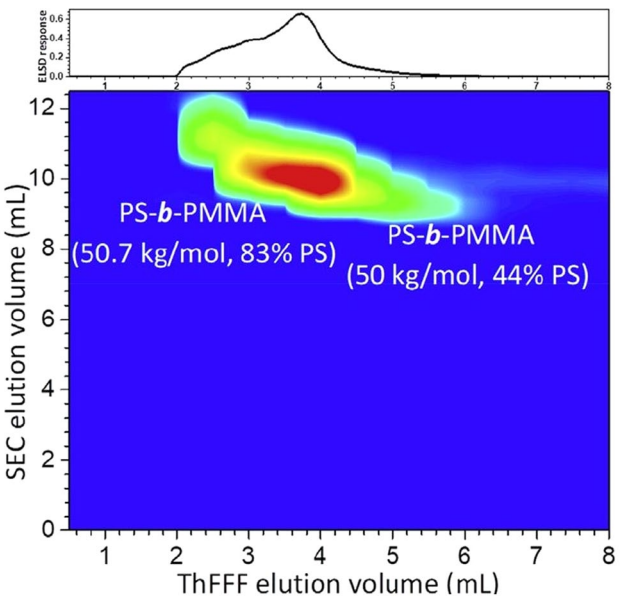

Fig. 3 SEC as the 2nd dimension in a 2D separation. ThFFF × SEC contour diagram of blend of two PS-*b*-PMMA copolymers. Fractogram, as determined using evaporative light scattering detection, is shown at top (Reprinted with permission from reference [19])

- Determination of fractal dimension across the MMD.
- Determination of persistence length and characteristic ratio.
- Determination of chemical and sequence length heterogeneity of copolymers.
- Determination of fundamental chromatographic information regarding retention, band broadening, and resolution.
- Determination of solution conformational entropy of mono-, di-, and oligosaccharides.
- Determination of core, corona, and total size of nanoparticles and quantum dots.
- Separation and characterization of individual components of blends.
- Contributing to a fuller understanding of the physicochemical phase space of copolymers and blends by adding a size-based separation dimension to 2D experiments.

Regarding the last point above, a recent Perspective on thermal field-flow fractionation (ThFFF) reviewed the online coupling of this technique with SEC [18]. Figure 3 shows how this coupling was employed in the analysis of two copolymers of polystyrene (PS) and poly(methyl methacrylate) (PMMA) of nearly identical molar mass but differing in monomeric ratio. ThFFF separated in the 1st dimension based on chemical differences, then SEC separated in the 2nd dimension based on the molar mass (size) of the individual ThFFF fractions [19].

Pitfalls and Challenges

Many of the caveats associated with performing SEC experiments and interpreting their results have been expounded upon in detail in references [1, 20–22]. By way of summary (and reminder), these include:

- *Forgetting that SEC separates on the basis of size, not molar mass*. Among other things this means that, for samples containing a heterogeneity of architectural structures, polymeric components, and/or copolymeric arrangements, any given chromatographic slice may contain macromolecules of different molar mass that are of the same solvated size as each other and thus co-elute. The phenomenon is not limited to SEC; it also extends to other size-based techniques such as hydrodynamic chromatography (HDC) and flow field-flow fractionation (flow FFF) [23, 24].
- *Over-reliance of peak-position (so-called "relative") calibration curves*, where the calibrants possess little, if any, architectural and/or chemical resemblance to the analytes [25]. This also includes reliance on pseudo-universal calibration curves, such as "Mark-Houwink" calibrations which, rather than using an online viscometer, apply supposedly known values for the

Mark-Houwink constants of the calibrants and analytes. These values are rarely known accurately, especially for the analytes.

- *Ease of concept and of incorrect execution*. As mentioned in the previous section, one of the great advantages of SEC is its conceptual and experimental simplicity, especially when only a single, concentration-sensitive detector is employed. This set-up, in combination with the application of peak-position, calibrant-relative calibration curves, is commonly employed in both academic and industrial labs for the determination of molar mass averages and distributions. It is a simple approach, easy to teach and to perform. MMDs and associated averages are generated in figure and table forms, usually with lots of unwarranted significant digits. Because only one detector signal is being integrated, precision is normally quite good. Unfortunately, in most cases, this just means that someone has become very good at reproducing inaccurate data, given the physicochemical dissimilarities between analytes and calibrants and the resultant inaccuracies in calculated molar mass averages and distributions. One may argue that there are cases where precision is more important that accuracy. While this is certainly so, the use of a single detector precludes the user from even being aware that important portions of the sample may not be included in the chromatograms. Therefore, one may ask what, exactly, has been precisely measured???
- *Assumptions with respect to column recovery.* It is often assumed, without any proof, that 100% of the injected sample has eluted from the column. To confirm this assumption, one needs to measure the specific refractive index increment $\partial n/\partial c$ of the sample by means of an off-line, batch-mode DRI experiment (or to measure the absorptivity a of the sample by the same type of UV/Vis experiment) [26]. It is otherwise impossible to know whether or not all the injected sample is contained within the chromatogram. Only under very specific and special conditions can one assume that 100% of the injected analyte is contained within a chromatographic peak [16].
- *Presence of other separation mechanisms.* As seen in Fig. 2 above, separation by an ideal, entropy-controlled mechanism is indeed possible in SEC. While most cases will not be this "perfect," they can be the result of a separation with minimal enthalpic contributions. There is, however, the possibility of alternative and/or additional separation mechanisms being at play (see Sects. 2.6.3 and 2.6.4 of [1], and also [27, 28]). The best ways to determine whether or not this is the case is with an on-line multi-angle static light scattering (MALS) detector, which can measure both M and size across the chromatogram. Retention-volume-invariance (invariance of V_R, as measured at the peak apex or center of gravitiy) as a function of varying flow rate is also a good indicator that extraneous separation mechanisms are not present.
- *Flow rate effects.* High flow rates can lead to on-column degradation, deformation, and turbulence, the last of which can have both adverse mixing effects and can also cause chain scission (i.e., degradation) [1, 27–32]. For large, linear macromolecules and/or polymers composed of relatively weak bonds as part of their structure, it may well be that flow rates as low as 0.1 mL min^{-1}, or even lower, are necessary. This is discussed more in the last section of this paper, within the context of ultra-high-pressure SEC. For macromolecules where even a portion of the MMD exceeds 1×10^6 g mol^{-1}, it is recommended to analyze the polymer at decreasing flow rates (or, more accurately, at decreasing linear velocities), until no change is observed in the chromatogram. For these large macromolecules, it is also recommended to use columns with the largest packing particle size available (covering the molar mass or, better, size range of interest, of course), to minimize interstitial shear rates which, when high, can lead to on-column, flow-induced degradation.

The characterization of ultra-high-molar-mass macromolecules, traditionally understood as those with M » 1×10^6 g mol^{-1}, has generally been regarded as a challenge. However, SEC can often effectively deal with these polymers, especially branched ones and, when it cannot other, gentler techniques exist such as HDC or flow FFF.

What has emerged as a greater challenge than the above, especially but not exclusively for regulatory purposes, is the accurate quantitation of oligomers, of the oligomeric portion of a macromolecule, or of the oligomeric content in a sample. The concerns involved have been addressed in detail elsewhere [33]. This type of quantitation will usually require a good deal of preparatory chromatography; off-line determination of detector responses (e.g., off-line, batch-mode DRI experiments to determine the $\partial n/\partial c$ of each oligomeric and polymeric species in the sample); and careful analytical chromatography, to ensure that all of the sample has eluted and has done so by a predominantly size-exclusion mechanism, and for the accurate placement of baselines and integration limits.

Conclusions and Outlook

Size-exclusion chromatography still retains its position as the preeminent macromolecular separation technique. This is due not only to its ubiquity in academic, industrial, and government laboratories but also to the wealth of information it generates about macromolecules, oligomers, and nanoparticles, especially when coupled to a multiplicity of physical

and chemical detectors. It is almost inconceivable to synthesize a new polymer in the lab, or to produce a polymer at an industrial scale, without characterizing its MMD and associated statistical moments using SEC. With care, accurate data can be obtained, and conclusion reached regarding the chemical and physical composition of samples and how these parameters interact and depend upon one another as a continuous function of molar mass. All these are integral to our understanding of the processing and end-use properties of macromolecules.

The last decade has seen the advent of so-called ultra-high-pressure size-exclusion chromatography (UHP SEC), sometimes also referred to as ultra-high-performance SEC (conveniently, the same acronym applies in both cases). Columns packed with sub-2-μm particles are employed in conjunction with a specially designed LC system with minimal dead volume and capable of withstanding pressures of > 65 MPa. Purported advantages of this approach include great increases in speed of analysis and chromatographic resolution [34]. While these gains have certainly been demonstrated, a number of caveats and limitations of UHP SEC bear mention here.

The small particle diameters of UHP SEC column packings translate into these columns possessing very narrow hydraulic radii. This, in combination with the very large pressures employed, means that analytes are subjected to incredibly high shear forces in the interstitial medium. These forces can lead to both degradation and deformation of the macromolecules (see Sects. 2.6.3 and 2.6.4 of [1], and also [27, 28]). Deformation can lead to elution by alternate separation mechanisms, such as hydrodynamic chromatography or slalom chromatography. Degradation, which in UHP SEC can occur either in the interstitial medium and/or during passage through column frits [28, 35], can lead to erroneous results regarding molar mass averages, dispersities, and distributions, among others. To avoid these problems, low linear velocities are required, somewhat offsetting the gains in speed. Also, one should note that the limiting step in most macromolecular separations is not analysis time but the time required for full sample dissolution, which can take anywhere from hours to days (overnight dissolution of samples with M between 1×10^5 g mol^{-1} and 1×10^6 g mol^{-1} is considered standard). Regardless, UHP SEC has great promise for the analysis of macromolecules with $M < 2 \times 10^6$ g mol^{-1} and, especially, as a fast, size-based second dimension in 2D-LC experiments [36].

While one of the greatest challenges for SEC and, indeed, for all size-based separations is the accurate quantitation of oligomers, perhaps the greatest opportunity for growth lies in coupling SEC to enthalpically dominated separations such as temperature gradient interaction chromatography (TGIC [37]), liquid chromatography at the critical condition (LCCC [38]), gradient polymer elution chromatography (GPEC [39]), or thermal FFF [18], in the form of 2D set-ups, such as TGIC × SEC, LCCC × SEC, GPEC × SEC, or ThFFF × SEC (see chapter 14 in [1]). These types of arrangements, wherein samples are separated by chemical composition in the first dimension and these first-dimension fractions are subsequently separated by size in the second dimension, can provide a virtually complete picture of macromolecules and blends, yielding the combined MMD × CCD of a sample. Advances in our understanding of how to develop IPC methods and hopeful future developments in the area of IPC column technology will greatly aid in this regard [9]. In the interim, while interest in and practice of 2D-LC of macromolecules has increased in the twenty-first century, these remain niche methods, mostly the bailiwick of a few, select groups around the world.

As always, but now more than ever in the past, the need for education in this area remains paramount [40]. Practitioners need to be trained not only in the areas of fundamental and applied chromatography but also in polymer science to understand their analytes and in a variety of instrumental techniques, along with their physical underpinnings, to understand what is being measured, how it is being done, and what the associated caveats are. Government and industrial funding is greatly needed, to encourage young professors and their students to explore these areas and to thus create an also greatly-needed generation of well-trained macromolecular separation scientists.

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Declarations

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