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Advances in size-exclusion separations of proteins and polymers by UHPLC

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ABSTRACT

The separation of molecular compounds based on their capacity to access the intra-particle pore volume of chromatographic media, which is dictated by the relative size in solution of those compounds, has been commonly known as size-exclusion chromatography (SEC) or gel-permeation chromatography (GPC). Conventionally, these two terms have been applied to the analysis of biomolecules and polymers, respectively. Over the more than half-a-century history of size-based separations, there has been a series of advancements, starting from the earliest soft-gel particles and culminating within the past few years in the use of sub-2-µm particles in ultra-high-performance liquid chromatography (UHPLC). The intent of this review is to provide a concise synopsis of the advancements of both chromatography columns and instrumentation for protein and polymer size-based separations. Also, this review presents brief summaries of the application of UHPLC technology for these classes of analytes.

Contents

1. Introduction

Size-exclusion chromatography (SEC) and gel-permeation chromatography (GPC) are two names for the same technique, the only difference being application area. SEC is predominately used to describe size-based separations of biomolecules, while GPC typically refers to separation of synthetic and natural polymers.

In this article, we discuss some of the more recent trends in the area of SEC separations. Historically, the technique was considered to be a low-resolution, time-consuming separation method. Indeed, the peak capacity for an SEC separation is substantially less than a gradient elution analysis. In SEC, the entire separation occurs within one column volume, while a gradient separation can be tens of column volumes, which lead to over an order of magnitude difference in peak capacity between the different separation modes. The materials traditionally used for SEC were limited in mechanical strength, thus precluding their use at higher flow rates. But, despite its limited peak capacity and lengthy separation time, SEC still plays an important role in separation and characterization of

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proteins and polymers. In this article, we discuss some of the new trends in SEC column and instrument design that are improving resolving power and enabling faster separations.

The predominant use of SEC for the analysis of biotherapeutic formulations has been in the measurement of the levels of reversible self-associated or aggregated (non-reversible) soluble high-molecular-weight (HMW) biomolecule forms that may impact the safety and the efficacy of a product. The level and the valency of soluble protein aggregation are critical quality attributes (CQAs) that require monitoring for monoclonal antibody (mAb) preparations intended for human use. Low-valency (e.g., dimer) HMW levels provide insight into process and product stability, as aggregation, which may occur throughout the manufacturing process from cell culture through final drug product formulation, may indicate partial denaturation or other perturbations of protein structure [1]. Also, the stability of the drug product, with respect to aggregation, must also be thoroughly understood. It is also critical to elucidate the distribution of high-valency, multimeric HMW forms in protein biotherapeutic preparations, since these multimeric forms have been reported to elicit an immune response aggressively by engaging an immunological pathway that is independent of T-cell involvement [2–4].

The use of SEC as the most common method for the quantitation of HMW levels in biotherapeutics is principally due to the sensitivity, the reproducibility, and the relatively high sample throughput of these analyses. However, one of the primary limitations of SEC is the potential of the method to not provide an accurate representation of the HMW forms present in a sample due to filtration or non-specific binding of the HMW forms by the column [5]. As a result, a crucial aspect of developing a reliable SEC method for the analysis of a biotherapeutic is confirmation of the separation observed by one or more orthogonal methods, such as sedimentation velocity analytical ultracentrifugation (SV-AUC), dynamic light scattering (DLS), or asymmetric flow field flow fractionation (afFFF) [6].

For the polymer industry, SEC provides critical information about the chemical composition and molar mass distribution, and how the molecule is constructed. This information provides data that can be correlated with some of the physical properties of a material, such as tensile strength, elasticity, and adhesion. The raw retention-time data generated from a chromatographic profile are transformed into a molecular-weight distribution. This is typically done by creating a calibration curve using standards of a range of known molecular weights (MWs). Narrow-dispersity polystyrene is most commonly used, and the calibration curve can be adjusted for the polymer composition of interest. This may require use of multiple detectors, such as ultraviolet (UV), refractive index (RI) and viscometry.

2. Stationary-phase development for SEC

The first demonstration of SEC was reported more than 60 years ago in 1953 by Wheaton and Bauman [7]. The broad application of this size-based separation for the isolation of biomolecules would begin six years later when Pharmacia brought to market spherical porous cross-linked dextran particles, under the trade name Sephadex [8–10], which is still commercially available. The size of the pore network of these particles depends on the degree of crosslinking, thereby modulating the optimal size range of biomolecules that can be separated. Other current gel-based particles were also produced in this era, including polyacrylamide-based gels [11,12]. These materials were commercialized by Bio-Rad under the trade name Bio-Gel.

The first SEC chromatographic media developed for hydrophobic polymers was by Moore of the Dow Chemical Company [13]. By cross linking with different amounts of divinylbenzene, porous gels could be synthesized with differing mean pore size. By packing the 44–75-μm particles into a 0.305 inch I.D. x 12 foot long tube, separations could be achieved in under 3 h. This was a significant improvement in time savings compared to the 3–4 weeks of extensive sample work-up required at the time [14]. Moore coined the term "gel-permeation chromatography'" to describe the technique of SEC specifically for polymer separations. Waters Associates licensed the technology from Dow, and commercialized the Styragel product line.

One of the key features that made Sephadex and Styragels widely used was their minimal interaction with proteins and organic polymers, respectively. However, both types of media were limited in mechanical performance. Their low operating pressure precluded their utility at high flow rates, or in configurations that utilized small particles. Since it was inherently a low-resolution technique, often two or three columns were connected together, resulting in run times of 30–60 min. Also, the polystyrene resins could shrink substantially and swell in different mobile phases, which meant that solvent switching could not be readily be performed without compromising the mechanical integrity of the packed bed. Manufacturers thus provided columns stored in several different solvents to remove the risk of adversely impacting column performance via solvent switching.

While it was well understood that reduced particle sizes would provide higher efficiency separations, it would not be until 1972 that a 10-μm porous silica particle would be brought to the market by Waters under the trade name of μPorasil [15]. The strength and rigidity of this particle enabled the creation of stable packed beds capable of operating at several hundred bars pressure, and able to withstand the shear stresses of high flow-rate mobile phases.

C18-modified silica became the workhorse tool for modern reversed-phase HPLC (RP-HPLC). Size-exclusion columns were also developed using porous silica, and typically optimized for this application by increasing the pore volume of the media. The surfaces required modification to minimize the strong ionic interactions between proteins and the acidic surface silanols of the silica by derivatizing with hydrophilic silanes [16–19]. Further reduction in interactions could be obtained by addition of mobile-phase additives [20]. Significant success was achieved with the use of a diol functional group. Even though acidic silanols remained, and could lead to ion-exchange adsorption of the charged proteins, the interaction could be substantially mitigated by utilizing high ionic strength mobile phases [19]. To this day, a diol phase remains as the most predominantly used silica-surface modifier for SEC of proteins.

In the case of polymers, a short-chain hydrocarbon silane was typically used for non-polar polymer separations, while unbonded silica proved effective for many hydrophilic polymers. However, while silica-based SEC columns became widely used for the characterization of proteins, cross-linked styrene is still widely used for polymer separations in non-aqueous media. One reason for this is the difficulty in effectively mitigating the ionic and hydrogen-bond interactions between silica and polymer analyte with compatible mobile-phase additives.

More recently, porous hybrid organic/inorganic particles [21] were developed and utilized for SEC. In 2010, Waters Corporation commercialized its first SEC column offerings with diol bonding, specifically for protein characterization. Subsequently, columns were commercialized with a trimethylsilyl (TMS) surface modification, or unbonded, for organic and aqueous separations, respectively [22,23]. One key advantage of these particles over silica is the significantly lower acidity of the hybrid silanols [24]. Fig. 1 shows the differences in silanol acidity for silica and bridged-ethyl hybrid (BEH) particles, both bonded and unbonded [24]. Acidity of the BEH-silica is seen to be substantially less than that of the silica. By surface-modifying the BEH particles with diol or trimethylsilyl (TMS) groups, silanol acidity could be further reduced.

One important consideration in the design of chromatographic media for SEC is the pore volume of the particle. In SEC, the differential size separation occurs almost entirely within the

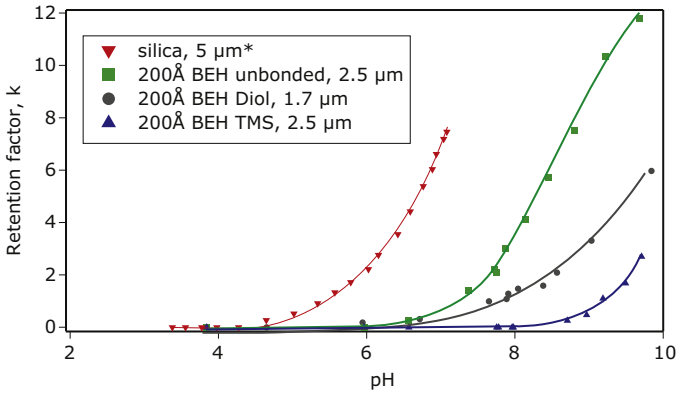

Fig. 1. Titration of silanol by plotting retention factor of nitrate ion as function of pH using method of Mendez et al. [25]. Mobile phase: 60% MeOH, 40% buffer (1 mM: sodium acetate, sodium phosphate, sodium carbonate, or sodium borate) Temp.: 30°C. Sample: 1.5 µL $LiNO_3$. Detection: Conductivity. *Data for silica (Waters Symmetry ®) adapted from Figure 3 in Mendez et al. [25], with permission from Elsevier. (Reproduced with permission from Waters Corporation).

intraparticle pores. Thus maximum separating power is achieved on particles with the greatest pore volume. However, this desire for high pore volume must be balanced against the mechanical strength requirements of the particle, as any increase in pore volume is at the expense of the solid structural component of the particle. Nonetheless, for the BEH particles, an increase of about 75% in pore volume was achieved while still maintaining the required mechanical rigidity for a 1.7-µm particle packed in a chromatographic bed and used at high pressures and shears [26,27]. These ultra-high-performance (or ultrahigh-pressure) liquid chromatography (UHPLC) columns provide significant gains in chromatographic efficiency when coupled with the appropriate UHPLC instrumentation.

Recently, monolith technology was demonstrated for SEC separations. For example, Li et al. [28] performed separations of protein mixtures in 30 min using a 23 cm x 150 µm capillary monolithic column comprised of poly(ethylene glycol methyl ether acrylate-co-polyethylene glycol diacrylate). Viktorova et al. [29,30] demonstrated the separation of up to 20×10^6 Da polystyrene on a monolithic divinylbenzene capillary column. One limitation of monolith technology is that the mesopore fraction of the column is typically substantially less than the intraparticle porosity of a bed packed with porous particles. This means that substantially longer column lengths are required for monoliths to achieve pore volumes similar to those of packed beds, resulting in longer separation times.

3. UHPLC instrument design for size-exclusion separations

The chromatographic efficiency of a peak that one observes is a result of both the column and the system. Ideally, one would like the system contribution to band spreading to be negligible compared to the band spread resulting from the chromatographic column. Modern UHPLC instrumentation is designed to add minimal dispersion to a chromatographic peak on a 2.1-mm ID column. This is typically the case for traditional adsorption modes of chromatography, such as RP, ion exchange, and normal phase. The process of adsorption within the column will broaden the peak, so demands on the system are reduced. The impact of retention on peak width is discussed in a related article in this issue [31], where the intrinsic peak variance is noted to be directly proportional to $(1 + k)^2$. UHPLC instruments were designed to add minimal system contributions to band broadening for retention factors greater than about 2. In the case of SEC, where there is no adsorption, the retention factor is zero, and the intrinsic peak width will be at a minimum. The peak variance obtained in SEC is thus seen to be almost an order of magnitude smaller than in adsorptive LC with a k of 2. Thus, to compensate for this, commercial size-exclusion UHPLC (SE-UHPLC) columns are provided with a 4.6-mm diameter in order increase the intrinsic peak variance, as this is proportional to the fourth power of the column diameter.

The extra-column dispersion of the injected sample can lead to significant losses of separation efficiency and undesired peak tailing [32–34]. These losses in efficiency can be introduced by unswept volumes in the autosampler, detector, and the tubing and end connections.

Another key attribute in instrument design is the compatibility of the system with mobile phases commonly used for SEC separations. For proteins, these are typically aqueous buffers with high salt concentrations. The chromatographic system used must be tolerant of the high-salt-concentration buffers used for these methods in addition to being biocompatible in order to minimize the formation of metal-protein adducts or protein-surface interactions. The wetted surfaces within chromatographic systems used for protein characterization are typically constructed of titanium, biocompatible polymers (e.g., PEEK) or biocompatible alloys (MP35N). For compatibility with polymer solvents, the system must be compatible with the broad range of non-aqueous solvents for dissolution and separation, often with aggressive/corrosive mobile -phase additives. These solvents must be delivered at pressures up to 1000 bar, without deleteriously affecting the flow delivery, seals and valves. Some of the solvents used for low-pressure GPC mobile phases may be limited due to their physical properties. For example, at room temperature, DMSO solidifies when subjected to pressures of about 500 bar. It is possible to use additives to depress the freezing point of DMSO, but this may induce adsorption or precipitation of the polymer of interest.

For polymer characterization, flow rate, precision and accuracy are critical to obtaining quality data. Because retention-time data are converted to MW, precision of the LC pump correlates directly with the precision of the molecular-weight distribution.

Recently, in 2013, Waters Corporation commercialized the Acquity APC® UHPLC, which was a system specifically designed for polymer separations. The isocratic system was designed to have low system dispersion. The materials contacting the fluidic components were chosen to be compatible with a wide range of mobile phases typically used for polymer characterization by SEC [23,35,36].

In SEC, a number of different detectors are used for characterization of polymers and biomolecules. For the analysis of proteins, peptides, and related compounds, UV absorbance detectors are most commonly used. A wavelength of approximately 280 nm provides good sensitivity for proteins and peptides that have amino acids tryptophan or tyrosine as part of their primary structure. However, disulfide bonds also absorb at this wavelength, and the molar extinction coefficient of this moiety is significantly lower than that of tryptophan or tyrosine [37]. The UV-absorbance band of the amide peptide bond (214–220 nm) can also be used and provides improved sensitivity over UV absorbance at 280 nm. However, this lower wavelength is more prone to baseline noise due to light scattering and may limit the use of some mobile-phase components. UV absorbance at 260 nm can be used to detect oligonucleotides separated by SEC. In the event that sample components interfere with protein detection by UV absorbance, the intrinsic fluorescence of these biomolecules can be used to advantage [38,39]. For the detection of polysaccharides, which have no chromophores, refractive-index (RI) detectors can be used [40]. Also, evaporating light-scattering detectors (ELSDs) have been commercially available for UHPLC use for several years.

In addition to using orthogonal methods, such as SV-AUC or DLS, to confirm the results observed by SEC indirectly, as previously noted, the direct characterization of the peaks separated by SEC is commonly performed using multi-angle light-scattering

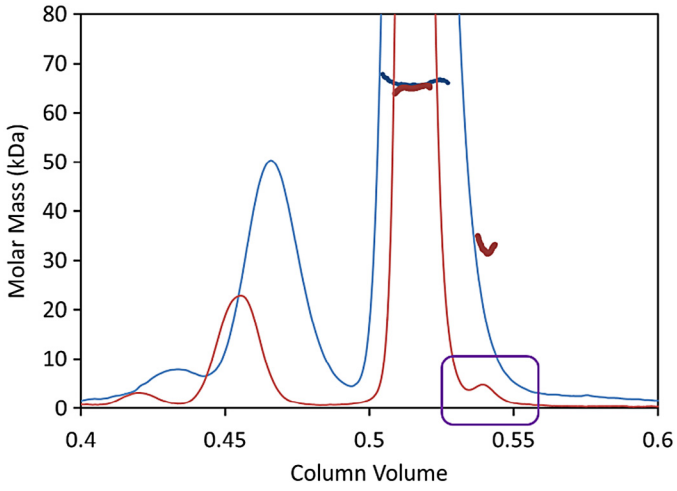

Fig. 2. Light-scattering data and measured molar mass for bovine serum albumin separated using UHPLC columns and instrumentation (red) and by standard HPLC columns and instrumentation (blue). Chromatographic conditions: Mobile phase 125 mM NaCl, 50 mM phosphate, pH 6.7; Temperature: 25°C. For UHPLC separation, detection was performed using a Wyatt µDAWN™ 660 nm, while HPLC separation was performed using a Wyatt miniDawn™ system. (Reproduced with permission from Wyatt Technologies).

(MALS) detectors. In conjunction with UV and/or RI detectors, absolute MW can be assigned [41,42]. More recently, low-dispersion RI detectors were commercialized in 2013 by both Waters and Wyatt [43,44]. In addition, Wyatt recently commercialized a low-dispersion MALS detector [45]. Fig. 2 shows overlays comparing the HPLC and UHPLC versions of the Wyatt MALS detector. The peak width is approximately 50% narrower on the UHPLC system, and is able to resolve a low-molecular-weight (LMW) constituent that could not be resolved using the HPLC detector.

Mass spectrometry (MS) detectors are increasingly being used for characterization of proteins and polymers [46]. However, there are particular challenges to coupling with SEC separations of proteins. Protein separations are typically performed using high concentrations of non-volatile salts, which can rapidly foul the MS source, and can also cause ion suppression [47]. SEC methods have been modified using denaturing mobile phases containing organic modifiers and with volatile buffers for use with MS detection [48–51]. For polymers, a distribution of charged species adds complexity to characterization of molar mass distribution. For this reason, matrix-assisted laser desorption/ionization (MALDI) is the most commonly used MS technique, as it generates primarily singly-charged species. However, MALDI is an off-line technique that requires deposition and evaporation of eluate onto a solid surface. Challenges remain in maintaining low dispersion from this process. As an alternative, Saucy et al. [52] have had success demonstrating the use of ^{210}Po as a means for charge reduction of electrosprayed polymers in aqueous media, but had less success with polymers in non-aqueous media. They had some success performing charge reduction for water-insoluble polymers when electrospraying in a solution of 5% trifluoroacetic acid in 1-methyl-2-pyrrolidone (NMP) [53].

Two-dimensional (2D) LC separations, which we discuss later in this article, often utilize NMR detection to obtain chemical composition information [54–56]. Reducing dispersion from transfer lines and the NMR flow cell presents challenges due to the distances needed to keep the LC instrument physically separated from the magnetic field.

4. Method development for biomolecule separations

Operationally, successful application of SEC for the analysis of biomolecules requires the consideration of two fundamental parameters. The first parameter is the use of an optimized mobile phase while the second is the extra-column dispersion of the chromatographic system. In order to achieve a separation primarily based on size or hydrodynamic radius of the analyte, the secondary interactions, both ionic and hydrophobic, between the biomolecule and the column must be eliminated or effectively minimized [57–59]. Not only can these interactions perturb the separation being attempted, resulting in observations of loss of protein recovery or deleterious changes in peak shape, they can also effectively alter protein secondary structure [60–62]. There are two principal types of ionic or electrostatic interactions that can affect SEC. The most readily noticeable of these is ionic adsorption, which occurs when the protein and chromatographic media have opposing charges and can result in low sample recoveries and peak tailing [63]. Less obvious is the phenomenon of "ion-exclusion", which can occur when the particles and the analyte have the same charge and will result in effectively excluding the analyte from the pores due to the ensuing repulsive forces. The chromatographic observation for this type of secondary interaction will be that the analyte will elute earlier than predicted based on its hydrodynamic radius.

Adjustments to the ionic strength and pH of the mobile phase are the primary means of reducing electrostatic interactions between the analyte and the SEC column [64,65]. While increasing the salt and/or buffer concentrations can minimize or eliminate undesired ionic interactions, there is also the possibility of introducing hydrophobic interactions with the diol ligands or other hydrophobic surfaces present in the column [65–68]. In these instances, using a more chaotropic anion, such as perchlorate, can be used to advantage [69]. Another approach to minimizing hydrophobic interactions is by adding an organic modifier, such as acetonitrile [70]. Another mobile-phase modifier that has been widely used to improve SEC protein and peptide separations is the basic amino acid arginine [62,71]. Arginine both stabilizes protein structure and prevents interactions between the protein and the column. While in the past there may have been concerns that arginine could be acting as a protein denaturant, as it has been observed to lower melting temperatures of proteins in solution, studies have shown otherwise [72]. As with other mobile-phase buffers, salts, and modifiers, it is important to use arginine of high purity in order to minimize chromatographic baseline noise to obtain optimal sensitivity. One of the limitations of arginine is that it absorbs and can therefore impair detection sensitivity at wavelengths below 220 nm.

5. SE-UHPLC applications

5.1. Biomolecules

There are numerous reported successful applications of size-exclusion HPLC (SE-HPLC) and many reviews and other publications have been devoted to this technology, some of which are in the References section of this review [20,69,73–76].

Certainly for the scientist who has initiated development of an SE-UHPLC method, much of the knowledge and many of the applications centered upon SE-HPLC can be directly applied to SE-UHPLC. By contrast, the number of applications reported for the use of SE-UHPLC is very limited, as this technology was only recently introduced (2010), and, currently, the only supplier of columns packed with sub-2-µm particles is Waters. However, commercially available SEC columns with 3-µm particles are available from Tosoh, Agilent, Phenomenex, Sepax, and Sigma-Aldrich. These columns provide some of the resolution, speed and sensitivity benefits relative to 1.7-µm particles compared to the classical SEC

columns with 5-µm and 10-µm particles. Both Waters and Thermo Scientific offer biocompatible UHPLC systems. As previously noted, UHPLC-compatible MALS and RI detectors are available from Wyatt.

The utility of SE-UHPLC separations has been realized in many areas of fundamental biochemistry research. In this capacity, these size separations have primarily been used to monitor the purity of laboratory-produced protein-related compounds [77–81]. In other examples, SE-UHPLC has been used as a purification step to purify cross-linked proteins in the study of cellular processes [82], and has also proved useful in protein-binding studies where differences in hydrodynamic radii between the reactant and the product can be used to advantage [83,84]. Proteomics is another area of research where the use of SE-UHPLC has been evaluated. Specifically, in the LC-MS mode, the utility of SE-UHPLC in a top-down proteomics strategy has been evaluated [85,86].

High-throughput and high-resolution separations, and the apparent molecular-weight range provided by SE-UHPLC have proved to be of significant value during the discovery and process-development activities associated with biotherapeutic proteins [87–91]. Also, SE-UHPLC has been successfully applied to the analysis of protein fragments [92], biotherapeutic leukocyte extracts [93], heparin [94], PEGylated proteins [95], and insulin and insulin variants [96,97]. An in-depth evaluation of the performance of SE-UHPLC was recently reported, and demonstrated that gains in sample throughput and the resolutions of high-efficiency separations can be achieved, when compared with SE-HPLC columns [98]. The authors also noted that the relative peak areas of the aggregate species of mAb panitumumab were observed to increase at higher temperatures and pressures, highlighting the importance of systematic method development and the confirmation of observed SEC profiles through the use of orthogonal methods [2].

In addition to these relatively traditional SEC applications, the characteristics of SE-UHPLC have been exploited in creative, novel methods. LC-MS separations under non-denaturing or native conditions have proved useful for the MS characterization of reduced mAbs, where the post-column addition of m-nitrobenzyl alcohol was used to improve electrospray ionization (ESI) and allow the MS identification of low-level species [99]. SEC LC-MS separations using direct ESI with a mobile phase of 25 mM ammonium acetate with 5% acetonitrile at a pH of 5.2 to evaluate the aggregation of a mixture of mAbs in stability studies were also reported [100].

Alternative separation strategies have been employed. The high sample-throughput solution using parallel interlaced SEC was reported as bringing the time of analysis for the aggregation levels of a mAb to below 2 min per sample [101]. An on-line 2D separation using an SE-UHPLC guard column (30 mm length) as a means of removing interfering small-molecule excipients in a sample prior to a mixed mode separation for the analysis of mAbs [102]. The reduced protein-column interactions and high efficiencies of the SE-UHPLC guard in comparison to SE-HPLC enabled the successful execution of this approach. The analysis of a mAb by a mixed-mode SEC and hydrophobic interaction liquid chromatography (HILIC) separation has also been reported [103]. In this example, the diol bonding and or the organosilica particle is being utilized as the ligand for HILIC interaction.

The high-efficiency separations provided by SE-UHPLC allow researchers to develop analytical SEC methods with greater resolution, improved sensitivity, and higher sample throughput than SE-HPLC methods. However, considerations of the performance of LC instrumentation and its implementation so as to minimize extra-column dispersion are critical in realizing the full potential of this technology.

5.2. Polymers

The first demonstration of the utility of UHPLC for polymer separations was in 2010 by Uliyanchenko et al. [104,105]. Using a 4.6 × 150 mm column packed with 1.7-µm 130-Å BEH C18, they were able to demonstrate separation of polystyrene standards with MW up to 50 kDa in less than 1 min. Separations were performed at a flow rate of 1.85 mL/min and an operating pressure of 660 bar. Columns were limited in pore volume, which reduced selectivity of the separation.

Janco et al. evaluated prototype columns packed with high pore -volume media for UHPLC separations by size exclusion [106]. They evaluated the impact of particle size on the polymer characterization. Using narrow-MW polymer standards with M_p of 11,600 g/mol, they compared the molar distribution on columns packed with 1.7-µm, 3.5-µm, 5-µm and 10-µm C18 particles. Fig. 3 shows the resulting chromatograms and molar mass. As particle size decreased, the calculated dispersity, Đ, defined as M_W/M_N, was found to become closer to the reported Đ value of 1.03.

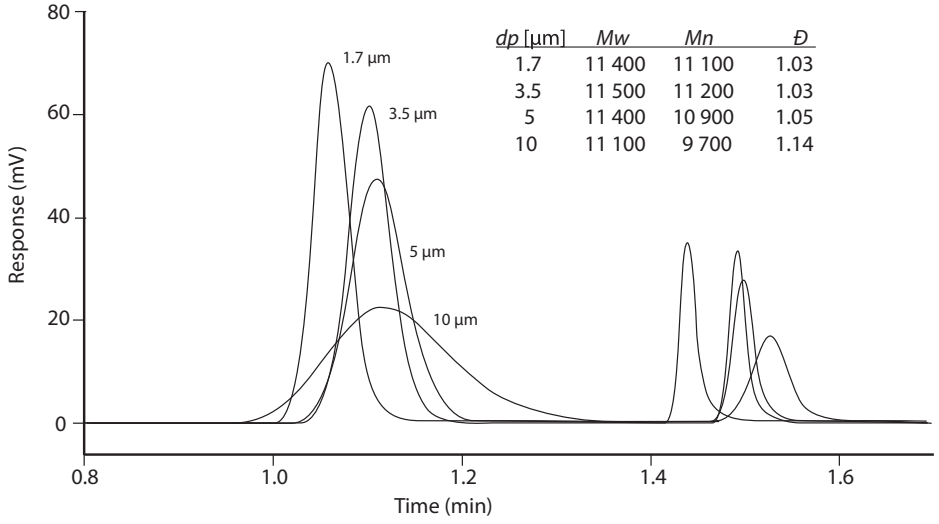

Fig. 3. Chromatograms of polystyrene standard (Mp 11 600 g/mol, Đ 1.03) obtained on XBridge® R C18 and Acquity C18 columns (4.6 × 150 mm) packed with different size particles: 1.7 µm, 3.5 µm, 5 µm and 10 µm. Mobile phase, THF; flow rate, 1 mL/min; detection, UV at 254 nm. {Reproduced from [106] under the terms of the Creative Commons Attribution Non-Commercial No Derivatives License (CC BY-NC-ND)}.

The impact of surface chemistry on polymer characterization was explored by Bouvier et al. [24]. As an enthalpy-driven process, retention should not be affected by temperature to a great extent. While the hydrodynamic radius can be impacted by temperature, the relative retention change is minor compared to enthalpic adsorption. Bouvier et al. [24] looked at a limited number of polymers on columns packed with both an unmodified and trimethylsilyl-modified on 200-Å BEH particles. They found that in a tetrahydrofuran (THF) mobile phase, the non-polar polymers saw comparable retention time decreases of about 1–2% when run at 50°C compared to 30°C on unbonded and TMS-bonded phases. Similar retention-time changes were observed on a corresponding divinylbenzene (DVB) column. However, polyethylene glycol was substantially more retained on the unbonded BEH phase at the lower temperature, and poly(4-vinylphenol) and poly(2-vinylpyridine) did not elute on the columns packed with the unbonded phase. Retention of these analytes was not affected by temperature on the TMS-bonded column. This indicates that the available surface of the unbonded BEH columns is able to interact by ionic and/or hydrogen bonding with these polar analytes.

5.2.1. Oligomer separations

Synthetic oligomers are used for numerous applications: lubricants, plasticizers, coatings, and intermediate prepolymers. It is desirable to be able to separate and to resolve as many of the individual components of the oligomer from each other, as that enables better identification and quantitation of the oligomeric component of the polymer or prepolymer. The number of oligomeric SEC applications has grown by two orders of magnitude in the past 30 years [32].

One key driver in characterizing oligomers is legal requirements for pre-manufacture notification (PMN) and for export/import regulations. The US Environmental Protection Agency (EPA) has exempted some classes of polymers from PMN, if the oligomer content is below a certain threshold [107,108]. The (e)(1) exemption pertains to polymers with M_n 1000–10,000 g/mol. Oligomers with molar mass <500 g/mol and 1000 g/mol must be <10% (w/w) and 25% (w/w), respectively. The (e)(2) exemption pertains to polymers with M_n above 10,000 g/mol. Oligomers with molar mass <500 g/mol and 1000 g/mol must be <5% (w/w) and 2% (w/w), respectively.

Oligomer separations by SEC present difficult challenges to chromatographic column and instrument design. The limited peak capacity of an SEC system precludes resolving all of the individual constituents of the oligomer. As MW increases, the difference in retention time between a polymer of *n* units in length from one of *n+1* units in length decreases. Above MW of ~1000–2000 Da, no observable resolution can be achieved in SEC between an *n*-mer and an *(n+1)*-mer. In the past few years, columns packed with smaller 3-µm and 5-µm particles were utilized for oligomer separations, primarily to achieve gains in speed and resolution. For separation of non-aqueous oligomers, porous styrene/divinylbenzene particles were traditionally used, and can typically operate at pressures less than 70 bar and deliver efficiencies up to 110,000 plates/m. The recent introduction of UHPLC to polymer characterization demonstrated an improvement in the resolving power of oligomer separations in significantly shorter run times. The use of 1.7-µm BEH particles enables faster flow rates on UHPLC instruments that can operate at pressures of 1000 bar. Fig. 4 shows a separation of oligomer constituents of a 374-Da polystyrene standard that can be achieved in less than 2 min [109].

Fig. 5 shows the impact of flow rate on chromatographic efficiency. In the case of oligomers, in which components are individually resolved, chromatographic efficiencies are up to 230,000 plates/m [109]. In the case of higher MW polymers, in which individual components are not resolvable, the chromatographic efficiency appears to be substantially less. However, in this case, the dispersity of the polymer has the most significant contribution to the peak width.

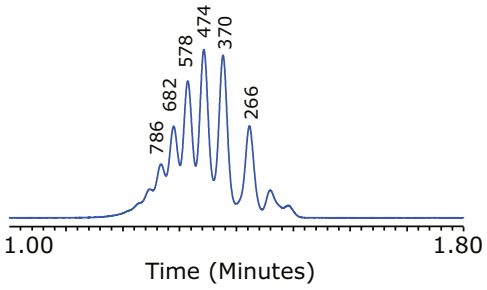

Fig. 4. Separation of polystyrene oligomers on a Waters APC 45, 4.6 × 150 mm, 1.7 µm in THF. Flow rate: 1.0 mL/min. (Reproduced with permission from Waters Corporation).

5.2.2. 2D separations

Complex polymers, such as blends and copolymers, present characterization challenges. They can contain distributions in MW and chemical composition that must be characterized. One such approach is to utilize comprehensive 2D separations (LC X LC), as discussed in a recent review article [56].

One common technique is to use LC under critical conditions (LCCC) as the first dimension [110]. In LCCC, conditions are chosen so that all constituents of the same composition elute at the same time, regardless of MW. Separations can be performed both off-line and on-line, but typically require several hours for complete analysis due to the time constraint of the second dimension, so the technique is impractical for routine use.

Recently, UHPLC-SEC was employed in the second dimension, with individual run times of less than 1 min, and total 2D separation occurring in 22 min [111]. This was demonstrated for the separation of polymethacrylate (PMMA) and polybutylmethacrylate (PBMA) copolymers. LCCC was employed in the first dimension, first to elute PMMA homopolymers, followed by an acetonitrile/THF gradient, providing a separation by chemical composition. SE-UHPLC was employed in on-line mode in the second dimension,

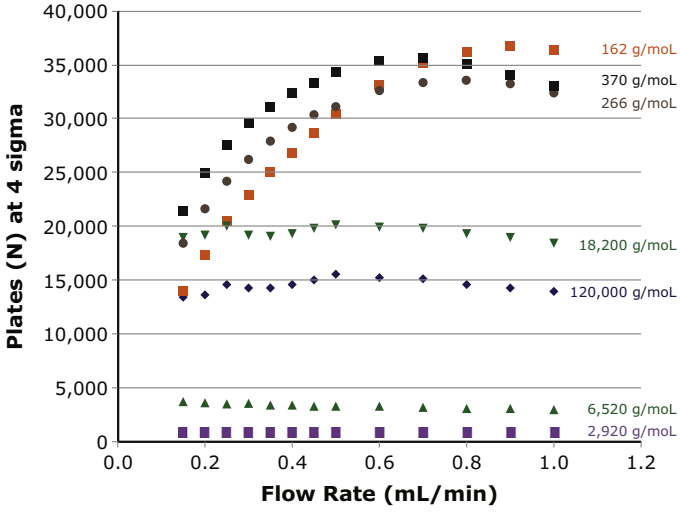

Fig. 5. Impact of flow rate on observed chromatographic efficiency for polystyrene standards. Column: Waters Acquity APC 45 XT, 4.6 × 150 mm; Mobile phase: THF; Temp: 25°C. (Reproduced with permission from Waters Corporation).

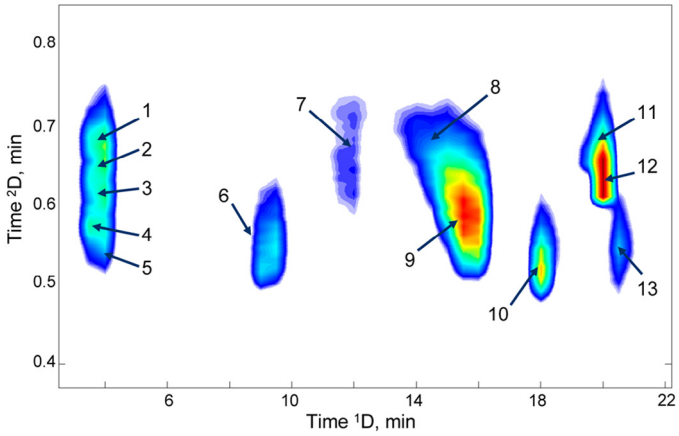

Fig. 6. Two-dimensional separation of PMMA and PBMA homopolymers and copolymers. First-dimension separation was performed on three Waters Acquity UPLC C18 columns connected in series, 2.1 mm x 250 mm total length. Gradient: 5 min at 15.5% THF in acetonitrile, followed by a 17-min linear gradient to 80% THF. Flow rate: 0.2 mL/min. Second dimension performed on an Acquity C18, 4.6 × 150 mm at a flow rate of 2 mL/min, in a THF mobile phase. {Reprinted with permission from [112], ©2012 American Chemical Society}.

providing the size-distribution information. Fig. 6 shows the results of the comprehensive separation.

Another approach, using conventional SE-HPLC as second dimension utilized high temperature to decrease mobile-phase viscosity and increase analyte diffusivity. This enabled faster separations with minimal degradation in chromatographic fidelity [111]. A 2D separation was performed, with second dimension runs of 1.6 min, for the analysis of polystyrene with different functional groups, and polystyrene-polyisoprene-polystyrene triblock copolymers.

One limitation in the use of multi-dimensional separations is the mobile-phase compatibility of the two techniques. When SEC is used in the second dimension, it is highly desirable for the sample diluent from the first dimension to be of sufficient strength for the analyte to be unretained on the stationery phase. Otherwise, adsorption during loading could impact the integrity of the peak and result in peak splitting [113]. Conversely, if adsorption chromatography is used for the second dimension, a weak solvent is needed for sample loading to concentrate the band. Peak spreading due to injection solvent can be mitigated by using smaller injection volumes. Alternatively, adding a make-up solvent and mixing tee could provide improvement, but at the expense of increasing the complexity of the system.

6. SE-UHPLC for HMW polymer characterization

HMW polymers are subject to shear stresses that can lead to deformation or shear [114]. As shear stresses are induced, the polymer can transition from a random coil to a stretched form. The extent of stretching can be characterized by the Deborah Number, a dimensionless number that represents the ratio of hydrodynamic forces to Brownian forces [115].

Both Uliyanchenko et al. [114] and Janco et al. [106] explored the effect of UHPLC on shear. Both groups found no shear-induced degradation of polymers up to 2–3 MDa. Slalom effects were observed for the HMW polymers, resulting in an increase in retention time. However, the slalom effects could be reduced by operating at lower linear velocities. Uliyanchenko found that shear-induced degradation could be induced for a 13-MDa polystyrene, but this could be avoided by operating at low linear velocities.

7. Benefits of UHPLC for size-based separations

SEC is an inherently a low-resolution technique, particularly when compared to other modes of chromatography. SEC separations are performed within one column volume, while isocratic and gradient elution chromatography use multiple column volumes to perform the separation. In the case of gradient separations, where band spread within the column is minimized, peak capacity can be more than an order of magnitude greater than in SEC [116,117]. However, SEC offers substantially improved selectivity over other separation modes when the primary characteristic being evaluated is size distribution. For example, determination of the extent of protein aggregation or the MW distribution of a polymer is most effectively provided by SEC.

The main utility of SEC is in the separation of large polymers and biopolymers, which have inherently low diffusivities. The resulting slow mass transfer of these analytes in and out of the stationary-phase pores limits the speed at which separations can take place.

Significant efforts have been made over the years to try to speed up or to increase the sample throughput of SEC separations [101,106,118–122]: by using higher flow rates, shorter columns, changing column aspect ratio, and performing staggered injections. However, the first three approaches result in decreased resolving power, while the last approach can add significant complexity to the chromatographic instrumentation.

The introduction of low-dispersion SE-UHPLC instrumentation and columns enables one to achieve faster separations without sacrificing resolution, by reducing particle size and column length, and maintaining the same L/d_p ratio. As discussed earlier, the success of this approach depends on using high pore-volume particles that have the requisite mechanical strength to maintain their integrity under high shear conditions.

Thus, speed is the primary benefit provided by SE-UPLC. By using a column packed with 1.7-µm particles instead of 5-µm particles, one can demonstrate that equivalent efficiency can be obtained in about one-ninth of the time. If one maintains the same L/d_p ratio, the approximate three-fold reduction in particle size enables a corresponding three-fold reduction in column length. In addition, the flow rate needs to be adjusted, since the optimum flow rate is inversely proportional to the particle size [123]. This combination of faster flow rate and shorter column length is what provides the nine-fold increase in sample throughput without sacrificing resolution.

In cases where even more resolution is needed, longer columns can be used, or multiple columns can be banked together to provide improved resolution without needing excessive run times. Since resolution is proportional to the square root of L/d_p, one would expect a 70% improvement in resolution for columns of equivalent length containing 1.7-µm *versus* 5-µm particles.

8. Conclusion

The benefits of enhanced chromatographic performance obtained with UHPLC were recently extended to separations by size exclusion, which has characteristics that place stringent demands on column and instrument design for UHPLC performance.

The dispersion requirements for SE-UHPLC are substantially more stringent than adsorption modes of chromatography, as the column contributions to band spread are at their smallest. In the past, column design suffered from several limitations:

- low-strength sorbents that could not operate at high pressures;
- swelling/shrinking when exposed to different mobile phases; and,
- adsorption to chromatographic media, particularly silica, which contained acidic silanols.

Recent advances in chromatographic column development have provided high-strength, high-pore-volume chromatographic media with low acidity. Surface modification has further reduced silanol acidity. Diol-bonded media have provided minimal interactions towards proteins using appropriate buffered aqueous mobile phases. Unbonded and TMS-bonded media can be used to perform effective size-based separations in aqueous and non-aqueous mobile phases, respectively.

While low-dispersion UHPLC UV and ELSD detectors have been available for the past decade, additional UHPLC-compatible detectors, such as RI and MALS, are beginning to be commercialized and can maintain the chromatographic integrity of these high-performance separations. MS detectors are successfully being used in conjunction with SE-UHPLC. By using volatile mobile phases, proteins have been effectively characterized with this powerful tool. We expect that SE-UHPLC separations of polymers will also benefit from MS and NMR detection, although challenges remain in interfacing these detectors with the separation to maintain low dispersion. Also, for MS, reducing charge distribution remains a challenge.

2D separations are expected to benefit greatly from SE-UHPLC. Chromatographic fidelity can be maintained for rapid SEC separations, and we expect the time required for comprehensive 2D separations to be reduced greatly from several hours to 30 min or less.

Even though the first commercial UHPLC columns for SEC were developed only four years ago, a number of protein-separation applications have already been developed, demonstrating the benefits of speed, resolution and sensitivity compared to conventional SE-HPLC. With the recent introduction of a system and columns for polymer characterization, the future also looks promising for characterization of these classes of analytes.

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