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Chapter 5

Size-exclusion chromatography

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5.1 INTRODUCTION

Size-exclusion chromatography (SEC), also called gel-permeation chromatography, molecular-sieve chromatography, or gel filtration, separates molecules according to their size. The term "gel permeation" is commonly used in polymer science for non-aqueous separations. Molecules of different size penetrate well-defined pores of column beads, where they are retained to various degrees. Smaller molecules are retarded on the column,
while larger ones are eluted more rapidly. As the retention time can be directly correlated with the size of molecules, this method is particularly useful for the determination of molecular weight. Since the current IUPAC recommendation still accepts terms such as molecular weight, molar mass, and molecular mass, they will equally appear in this text.

The use of size-exclusion phenomena for the separation of natural polymers had been already discussed in the late 1940s when materials, such as zeolites, charcoal, and ion-exchange resins were developed as sorbents. However, it was not until the middle of the 1950s that size-exclusion chromatography appeared in the literature [1,2], with starch as the column material. This was followed by a period of intensive activity at Uppsala University [3–8], where in 1959 Porath and Flodin [9] produced cross-linked dextran, which was commercialized by Pharmacia as Sephadex, and in 1962 Hjertén and Mosbach [10,11] developed cross-linked polyacrylamide (Biogel P) and agarose (Sepharose). During the same period, Polson [12] reported that agar was also a suitable column material for separating natural polymers. Since then, a large number of separation media have been developed, and these will be described in greater detail in this chapter. General reviews on SEC, including theory, equipment, and instrumentation can be found in Refs. 13–17.

SEC is now generally applicable to the separation of molecules in the range between 0.5 and 1000 kDa, but larger proteins or other giant molecules can also be separated [18]. Moreover, SEC can also physically separate folded, from unfolded macromolecules, particularly in the case of slow equilibria [19]. The major applications of SEC include the determination of molecular weight and molecular-weight distribution (polydispersity) of natural and synthetic polymers. Separation strongly depends on many factors, among them column packing, column dimensions, flow-rate, sample volume, and mobile-phase composition. These parameters may substantially contribute to the quality of separation and possible errors, such as band-broadening and decreased resolution. This effect was observed by Busnel et al. [20] with standards having very narrow molar mass distribution. Mapping of band-broadening was observed for different column sets. These aspects were also studied in detail by Ricker and Sandoval [21], who presented practical guidelines for the development of reproducible SEC methods, based upon optimized sample volume, flow-rate, column length, and mobile-phase conditions that reduce nonideal SEC behavior – parameters often ignored in this type of separations. Adjustment of these factors frequently results in more accurate elution times, more precise determination of molar mass, sharper peaks for improved resolution and shorter run times for increased throughput. In general, sample volume and flow-rate should be kept to a minimum for optimal resolution. Increasing column length improves resolution and may be achieved by connecting columns in tandem. Adjustment of the mobile-phase conditions can significantly enhance resolution, but results are difficult to predict, because unique sample properties play a major role in this interaction, as does the column packing. Whenever possible, ionic strength and pH of the mobile phase should be adjusted until the peak(s) of interest are eluted at the expected time and with good peak shape. Finally, the use of smaller-diameter columns (i.e., 4.6 mm rather than 9.4 mm ID) and packings of smaller particles (4–5 μm) may also be considered. These factors will be described below.
5.2 THEORY

SEC is defined as the differential elution of solutes, from a bed of a porous chromatographic medium, caused by different degrees of steric exclusion of sample molecules from the pore volume of the molecular size of solutes. Thus, in SEC, solutes are eluted strictly according to decreasing molecular size, and the maximum available volume for separation is equal to the total pore volume of the packing medium. The retention volume, \( V_R \), is given by

\[
V_R = K_D V_P + V_O
\]

where \( K_D \) is the distribution coefficient, i.e., the available pore fraction (ranging from 0 to 1), \( V_P \) is the pore volume of the packing medium, and \( V_O \) is the extra-particle void volume. The relative pore volumes of packing media vary from 50% for silica-based materials to over 95% for semi-rigid polymer-based media. Since the separation volume is limited in SEC, the calculation of peak capacity can be reduced to

\[
n_{R_s} = 1 + V_P / (V_t(N/16)^{1/2}/R_s
\]

where \( n_{R_s} \) is the number of peaks separated with a resolution of \( R_s \), \( V_t \) is the total liquid volume of the column (i.e., \( V_P + V_O \)), and \( N \) is the maximum number of theoretical plates of the column. The peak capacity of SEC columns is often cited as

\[
n = 1 + 0.2N^{1/2}
\]

However, this equation is valid only if \( N \) has the same value for all solutes, which is not the case for macromolecules. That is why Giddings [22] suggested that an average plate count could be assumed in the calculation. From these equations and the statements above one can conclude that the peak capacity of a SEC column is much smaller than that for many other liquid chromatography (LC) columns. There is also little or no advantage, with respect to separation efficiency, in using a smaller-sized packing material in SEC, since the pore volume then decreases and the column length is often shorter. The only gain would thus be in separation speed. This could, of course, be very important in multiple separations, where processing speed needs to be high. Another point to consider is that zone-broadening in SEC is diffusion-controlled. That is why factors affecting the total diffusion time (i.e., column length, the diffusivity of the solute, which is 10–100 times slower for macromolecules than for small inorganic molecules) and the diffusion distance (i.e., pore and particle size of the packing media) are crucial. Other factors to be considered include the sample volume, which should typically be smaller than 0.2% of the total bed volume in order to avoid peak-broadening. There are also some simple “rules of thumb” regarding sample concentration in SEC, which generally should not exceed ca. 70 mg/mL for a globular protein, 5 mg/mL for a dextran with an average molar mass of 100 kDa, and 10 mg/mL for a dextran of 10 kDa.

Practical aspects of SEC, including instrumentation and troubleshooting, have been described in a recent review by Titterton [23]. Since no gradients are used in SEC, the chromatographic equipment can be rather simple. For optimal results, packing of the column must be carried out very carefully. The volume of the loaded sample should not
exceed 5% of the column volume for preparative runs and 1% for analytical applications. For production-scale separations, column diameters up to 30 cm and gel-bed lengths up to 120 cm are recommended.

In protein separations, the resolution is not correlated with the total amount of protein loaded onto the column. Thus, highly concentrated protein samples will give the best separations. However, in the case of carbohydrates, resolution is likely to be affected by molecular interactions if the sample concentration is too high. It should be noted that SEC columns are not able to concentrate samples, in contrast to some other LC columns. Online sample treatment for or by column liquid chromatography has been discussed by Brinkman [24].

A recent review by Winzor [25] summarized the theory of SEC and described the development of chromatographic techniques for the determination of reaction stoichiometry and equilibrium constants for solute interactions of biological importance. Gel chromatography, affinity chromatography, and studies of interactions by biosensor technology were reviewed in detail. A general stochastic theory of SEC, which can account for size dependence on both pore ingress and egress processes, moving-zone dispersion, and pore-size distribution, has recently been developed by Dondi et al. [26]. A unified theory for gel electrophoresis and gel filtration was presented earlier by Rodbard and Chrambach [27], who later published on kinetics of hormone/receptor and antigen/antibody interactions. A quantitative theory for gel-exclusion chromatography also arose from the studies of Polson and Katz [28].

Calculations of the hydrodynamic permeability of gels and gel-filled microporous membranes have been published by Mika and Childs [29]. A model was developed by Rill et al. [30] to simulate SEC separations of globular proteins on templated gels. In this study, it was assumed that the partition coefficient for sieving of a protein is equal to the fraction of gel volume accessible to a sphere with a radius equal to the Stokes radius of the protein. An interpretation of virial coefficients, reflecting thermodynamic nonideality in incompressible solutions of a single macromolecular species for which there is no volume change on mixing, has been reported by Wills and co-workers [31]. The findings were discussed in relation to the results obtained by osmometry, isopiestic measurements, equilibrium dialysis, gel chromatography, and sedimentation equilibrium. This group has also reviewed the quantitative characterization of biospecific complex formation [32]. The merits of frontal gel chromatography, electrophoretic methods, and affinity chromatography were discussed and theoretical and experimental studies have been made on the advancing elution profile in frontal gel chromatography [33]. A study of multiple polymerization equilibria by glass-bead exclusion chromatography with allowance for thermodynamic nonideality effects has also been reported [34]. Thermodynamic nonideality and the dependence of partition coefficients upon solute concentration were studied by Minton [35]. A gel-chromatographic procedure that corrects for Donnan effects in studies of ligand binding was discussed by Jordan et al. [36]. The origin and consequences of concentration dependence in gel chromatography were reported by Nichol and co-workers [37] and Winzor and Nichol [38], while Hibberd et al. [39] discussed an experimental and theoretical investigation of boundary spreading in gel chromatography. An interesting paper has recently been published by Brooks et al. [40], where an alternate picture proposes that the partition coefficient can be calculated from a
thermodynamic model for the free energy of mixing of the solute with the gel phase. Size-dependent exclusion, caused by the unfavorable entropy of mixing, associated with the partition was predicted, the magnitude of the effect being modified by enthalpic interactions between the solute and the gel phase. This concept has been extended to describe the partition of macromolecules into a layer of terminally attached polymer chains, grafted onto a solid bead.

5.3 COLUMNS

Several excellent reviews summarize the properties and applications of various column packings, both inorganic-based and polymeric-based stationary phases [41,42]. Porous glass was one of the first new-generation rigid packings for SEC [43], as opposed to the soft gels. However, there were problems in its use, not the least of which was a tendency to irreversibly adsorb polar solutes, due to the presence of charged sites in the silicate matrix of the glass. Such packings were later superseded by silica-based materials, in which the charged sites had been deactivated by the use of an inactive bonded phase. Commercially available SEC particles are not homogenous in their size. Therefore, their separating capability is lower than expected. Ekman et al. [44] have described a simple and efficient method, called dried elutriation, to separate Sephadex beads of narrow size distribution.

Hjertén and Eriksson [45] studied the high-performance molecular-sieve chromatography of proteins on agarose columns, and investigated the relation between concentration and porosity of the gel. Curves showing the relationship between the logarithm of molecular weight and distribution coefficient were presented for proteins subjected to chromatography on cross-linked and non-cross-linked agarose gels of different concentrations. Plate numbers were determined for columns of 20% agarose at different flow-rates and bead sizes. Anspach et al. [46] subsequently performed comparative study of the application of the silica-based packings (Zorbax Bio Series GF 250 and GF 450 and TSK-Gel 3000 SW and SWXL columns) in the chromatography of proteins. It was found that reduction of the mean particle diameter of the silica-based packings in the SEC of proteins to about 5 μm generated the expected increase in column plate number over the traditional 10-μm SEC columns. Slightly lower column efficiency of the TSK-Gel 3000 SWXL compared with the GF 250 column was compensated by the fact that the phase ratio of the 3000 SWXL column is higher by a factor of two. Both columns showed nearly the same peak capacity of about 20–30 in this application.

Santarelli et al. [47] investigated dextran-coated silica packings for high-performance SEC of proteins. Porous silica beads have excellent mechanical properties, but non-specific interactions between silanols on the silica surface with the proteins require modification of these media before they can be used as stationary phases for SEC. Silica beads were coated with dextran, bearing a small number of positive charges in order to neutralize the negatively charged silanol groups. For this purpose, diethylaminoethyl-dextrans (DEAE-dextrans) with a relatively low percentage of dextran units, bearing DEAE functions, were layered on the silica beads. The effects of these packings on chromatographic performance were studied in order to determine the optimal conditions for the SEC of proteins. An investigation of the physical, chemical, and functional
properties of one of the first semi-rigid polymer packings for SEC, Sephacryl HR, was described by Hagel et al. [48]. High-performance SEC of some standard proteins, peptides, and amino acids on another hydrophilic packing material, obtained by chemical transformation of a cross-linked polystyrene/divinylbenzene copolymer, was also studied [48]. The characteristics of columns filled with 4- and 7-μm particles were compared. The influence of acetonitrile, 2-propanol and trifluoroacetic acid at various concentrations in the mobile phase on the chromatographic performance of the columns was investigated. A linear calibration graph, covering the molecular-weight range from 200 to 700,000, was obtained under optimal conditions [49].

The mechanical stability of the chromatographic media marketed as Fractogel EMD BioSEC, permits higher flow-rates than softer gels. This feature appeared to be helpful during the regeneration and equilibration of columns, as well as in chromatographic runs. The pressure stability also facilitates packing of larger columns, which are sometimes necessary for refining pharmaceutical proteins. High stability toward alkali treatment enables production-scale separations on Fractogel EMD BioSEC. The separation media that have been commercialized under the names Superdex and Superose are based on dextran, cross-linked with agarose to various extent. The name Superose applies to packings of artificially cross-linked agarose, as opposed to the older agarose media, cross-linked only by natural hydrogen bonding. This type of matrix has a very good stability over a broad pH range. A Superdex Peptide column is a variant capable of separating small molecules (peptides and single amino acids) within the 100- to 7000 Da range. Based on the improved performance in the speed of chromatographic separation on Superdex-type materials, compared to conventional media, such as Sephadex and Bio Gel, a rapid SEC method was developed for the separation and analysis of carrageenan oligosaccharides [50]. Vilenchik and co-workers [51] have recently presented evidence that useful microporous materials can be obtained from protein crystals (CLPC). The CLPC materials can be made chemically and mechanically stable and are capable of separating molecules by size, chemical structure, and chirality. This allows estimation of the apparent pore-size and pore-size distribution in solid and soft hydrated porous sorbents by SEC.

5.3.1 Monolithic materials

Monolithic materials are continuous rods of solid, porous polymers rather than separate beads. Molecular-weight determination for polymers can be improved by using complementary techniques, such as chromatography on the monolithic columns, thus providing valuable information on the composition of co-polymers [52]. Preparation of molded, porous, polymer monoliths with controlled pore structure was described by Peters and co-workers [53], and the history of the development of macroporous columns (though mostly concerned with ion exchangers) was published by Abrams and Miller [54]. The use of monolithic high-performance SEC media for screening of polymers was reported recently [55]. The rapid development of this type of columns was intensified following publications by Hjertén’s group [56,57]. Another methodology for the preparation of polymer gels with variable pore architecture was demonstrated by Antonietti et al. [58]. Electro-osmotically driven SEC of polystyrene standards with molecular weights of up to $10^6$ Da has been described by Peters and co-workers [59]. Mayr et al. [60] studied the
influence of polymerization conditions on the separation of proteins by inverse SEC. SEC was also applied to control the pore-size distribution of the monolithic poly-TRIM grafting substrate [61]. Separation of styrene oligomers by several modes with this technique has been reported [62]. It seems that further miniaturization of capillary columns (below 300 μm ID), filled with "traditional" gel-type stationary phases will eventually be replaced by the use of monolithic columns, as they are much easier and faster to prepare, providing suitable reproducibility and control over a broad mass range.

5.3.2 Capillary columns

Capillary SEC affords an alternative separation strategy for microscale purification [63–71]. Capillary SEC columns are best suited for direct coupling to electrospray-ionization mass spectrometry (ESI-MS), because they have comparable flow-rates that can be delivered to the ESI source without splitting. Because no split is necessary, the sensitivity of such separations is much higher, and less material can be applied on the column. For example, scaling down the diameter of a Superdex Peptide column from 10 to 0.3 mm causes a significant decrease in sample consumption from 5 μg to 50 ng. Various successful applications of SEC microcolumns have been described [72–74]. Micro-SEC separations can also be indirectly linked with matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF-MS) by using robotic interfaces [75]. Other references to these topics are cited in Secs. 5.5 and 5.7.

5.3.3 Microsystems

There are still very few references dealing with microchip (micro-Total Analysis Systems, μTAS) technology, used together with packed channels, though there is a rapidly growing number of papers describing capillary electrophoresis on microchips in conjunction with MS and other detection techniques (Chap. 11). Rapid progress in the development of monolithic media can extend these applications [76], because preparation of channels filled with these stationary phases is much easier than filling them with "classical" column packings. Two papers giving a general and complete overview of the present status of μTAS were published recently [77,78]. A prototypic microchip has been constructed for the size separation of macromolecules and particles by hydrodynamic chromatography [79]. The device has been applied to size characterization of macromolecules.

5.4 MOBILE PHASES

SEC is a very mild separation method, because buffer systems are used as the mobile phase. Thus, optimal conditions with respect to the stability of solute molecules can be selected. This aspect was extensively studied by Garcia et al. [80]. Aqueous SEC was used to analyze the elution behavior of several standard ionic polymers as a function of the pH and ionic strength of the eluent. Two organic-based hydrophilic packings, Spherogel TSK PW4000 and Ultrahydrogel 250, were tested in order to select the optimal conditions for
macromolecular separation. Deviations from ideal elution behavior have been attributed to ion-exclusion and hydrophobic effects, as a consequence of the repulsive or attractive interactions between the ionizable groups of the poly-electrolyte and the residual surface charge of the support. Quantitative evaluation of elution volumes of poly-electrolytes in salt-containing eluents was performed, taking into account electrical double-layer effects and the effective radius of poly-ions, and assuming that poly-electrolytes behave as rigid hydrodynamic spheres and that the geometry of gel pores is cylindrical [81]. Reported data on the elution of sodium polystyrene sulfonate and polyglutamic acid from both organic- and silica-based packings were used to test the accuracy of the predictions [82].

The effect of column and mobile-phase modifications on retention behavior in SEC of polycyclic aromatic hydrocarbons on poly(divinylbenzene) has also been described [83]. Molecular-mass distribution analysis of ethyl(hydroxyethyl)cellulose was studied by SEC with dual light-scattering and refractometric detection [84]. The polymer aggregates showed variations in behavior that were dependent on the flow-rate and ionic strength of the mobile phase. Molecular characterization of cellulose is technically difficult because of the limited number of appropriate non-degrading solvents. SEC of cellulose was described by Hasegawa et al. [85]. Schult et al. [86] applied SEC on macroporous, monodisperse poly(styrene/co-divinylbenzene) particles and LiCl in N,N-dimethylacetamide to dissolve cellulose. Phillips and Olesik [87] performed studies of LC with enhanced-fluidity liquids. This new technique has led to the continued development of LC at the critical condition (LC-CC) or liquid chromatography at the critical adsorption point (LC-CAP). LC-CC allows isolation of one area of the polymer matrix so that other areas of the polymer can be probed with size-exclusion or adsorptive chromatographic modes.

SEC analysis in aqueous systems has provided information on solubility and aggregation of xylans [88]. Xylan samples from different sources were investigated, using a multi-detector SEC system with two chromatographic column sets and mobile phases consisting of dimethylsulfoxide (DMSO)/water mixtures in various proportions. Molar-mass distribution could be best analyzed by using a mobile phase of DMSO/water (9:1) with the addition of 0.05 M LiBr, a system offering good solubilization of the polymers and an effective chromatographic separation. The use of tetrahydrofuran (THF) as mobile phase in the SEC characterization of a liquefaction extract and its hydrocracking products has been found to cause partial loss of sample and to give anomalous results [89]. However, the problem was solved by using 1-methyl-2-pyrrolidinone as the mobile phase. Li and co-workers [90] have described a method for the determination of iodide in seawater and urine by SEC with the iodine/starch complex. Iodide was converted to iodine, then sequestered with starch, and separated from the matrix, using a Shim-pack DIOL-150 size-exclusion column with methanol/0.01 M aq. phosphoric acid (1:9) as mobile phase at 1.2 mL/min. Batas and Chaudhuri [91] have described a mechanism for SEC-based protein-refolding, another novel effect in SEC. This model considers the steps of loading denatured proteins onto a column and its elution. The predictions were compared with results obtained by SEC of lysozyme on Superdex 75 HR with a refolding buffer. The main collapse in protein structure occurred immediately after loading, where the partition coefficient increased from 0.1 for unfolded lysozyme to 0.48 for the partially folded molecule. The use of a refolding buffer as the mobile phase resulted in complete refolding of lysozyme. The effect of mobile phase on the oligomerization state of α-helical
coiled coil peptides during high-performance size-exclusion chromatography (HPSEC) was studied by Mant et al. [92]. HPSEC appeared to be useful for examining both the oligomerization state of coiled coils and the stability of such motifs, due to facile manipulation of the mobile phase and the lack of interaction of the peptide solutes with the stationary phase.

The influence of three experimental parameters – temperature, pH, and ionic strength of the eluent – on the retention of heparin samples and polysaccharides as calibration standards in SEC was investigated by Bergman et al. [93]. Silvestre et al. [94] separated protein hydrolysates on poly(2-hydroxyethylaspartamide)-silica columns with 0.05 M formic acid as eluent. Concentrated formic acid (70%) was applied for elution of Aβ fragments present in human brains, and a Superose-12 column was used without significant deterioration of the stationary phase due to the acidic eluent [95]. Nylander et al. [96] reported that the mobile-phase composition may influence adsorption and separation of neuropeptides on the Superdex-75 column. These studies were extended by Hedlund and co-workers [97]. In general, both acetic and formic acids, commonly used in chromatography and mass spectrometry, contain C=O groups in their structures. The double bond accounts for a high absorbance in the UV detectors up to 240 nm, a factor that should be carefully considered in the detection of, e.g., a peptide bond (usually at 210–214 nm). Under overload conditions, the resolution in SEC of proteins can be compromised due to non-uniform flow, caused by the viscous-fingering flow instability. In the work of Fernandez et al. [98], the non-uniform flow under these conditions was analyzed by numerical simulation and magnetic resonance imaging, and a new column design was postulated. Another recent aspect of protein chemistry was investigated concerning speciation of zinc in complexation with proteins of low molecular mass that occurs in breast milk and infant-feeding formulas. SEC was used in conjunction with atomic-absorption spectroscopy [99]. After ultracentrifugation of the milk, the sample was injected into a TSK-Gel G2000 glass column and eluted with 0.2 M NH₄NO₃/NH₄OH (pH 6.7). This was followed by inductively-coupled plasma mass spectrometry (ICP-MS) analysis of the eluate.

An analytical HPLC method has been reported for the simultaneous measurement of low concentrations of dextran-methylprednisolone succinate and its degradation products, methylprednisolone hemisuccinate (MPS) and methylprednisolone (MP) [100]. The analytes were detected at 250 nm after resolution on a size-exclusion column with a mobile phase of 10 mM KH₂PO₄/MeCN (3:1) at a flow-rate of 1 mL/min. The resolution of MP and MPS peaks was substantially affected by the pH of the mobile phase; the degradation products were not resolved at pH 3.4. Hyaluronic acid has been analyzed by HPSEC on a TSK-Gel 6000 PW column and eluted with 100 mM NaNO₃ [101]. A method for analytical and preparative SEC of large, water-insoluble, protected peptides in an organic solvent was developed by Karnoup and co-workers [102]. This method was applied to the analysis and separation of protected synthetic peptide tandem repeats and to control the peptide fragment coupling. Toyopearl HW-40, HW-50, HW-55, and HW-60 columns of fine grade were used, and the selectivity of each sorbent, as well as the chromatographic behavior of the peptides were examined. Fractionation ranges of these gels in N,N-dimethylformamide (DMF) were shown to extend over much
smaller molecular masses (ca. 400–14,000 Da) than those of the same gels applied to separate proteins in aqueous buffers (100–1,000,000 Da).

Mobile-phase composition, pH, and flow-rate can significantly affect separation in SEC. Special care must be taken to optimize separations before sample application in order to minimize variations in retention times and possible sample loss due to, e.g., non-specific adsorption on the gel. In certain cases, addition of NaCl is necessary to reduce non-specific interactions, but salts may influence further analysis by mass spectrometry. Moreover, phosphate buffers are not recommended, as they can polymerize in heated capillaries (ESI-MS), thus forming insoluble polyphosphates.

5.5 DETECTORS

Detection of separated components is a crucial step in the chromatographic procedure, as it provides information on the character of the molecules. The major features of an effective detector are: selectivity, specificity, sensitivity, stability, and linearity. It is also desirable that the detector will not destroy the sample, but this does not apply to some mass spectrometers, e.g., those equipped with an ESI ion source. Samples from MALDI or Fast Atom Bombardment (FAB) targets can possibly be recovered for further analysis, such as radioimmunoassay (RIA), radio-receptor assay (RRA), enzyme-linked immunosorbent assay (ELISA), etc. Anyhow, modern mass spectrometers utilize only minute amounts of sample, and can give unambiguous results that outweigh these disadvantages. The characteristics of the ideal detector for liquid chromatography have been summarized by Lemiere [103], and several fundamental reviews on this topic have also been published in recent years [104–108]. The "ideal detector" should be able to identify the molecule of interest, quantify it (possibly in the presence of other components), and provide complete structural information for the unambiguous identification of the isolated compound. From this point of view, mass spectrometry, nuclear magnetic resonance (NMR), and Raman spectroscopy, including the flow-injection surface-enhancement Raman scattering (SERS) variant [109–111], are the methods of choice for either on-line or off-line detection. These are often used in conjunction with other techniques, such as RIA, ELISA or other, less specific detectors (UV, IR, electrochemical detectors). For example, Fourier-transform infrared (FTIR) spectrometry in the mid-infrared region is becoming more and more important in SEC. It is a powerful and potentially very widely applicable method for obtaining information on the chemical functional groups in each molecular-size fraction [112]. Quantitative evaluation of polymer composition across the SEC chromatogram can provide a more accurate characterization of heterogeneous polymer samples, which is necessary for problem solving and material specification. Detection limits, dilution factors, and technique compatibility in multi-dimensional separations have been discussed by Schure [113].

SEC with coupled multi-angle light-scattering- (MALS), and differential-refractometry detectors have been used to obtain molecular mass and radius of gyration distributions of polydisperse polymer samples [114]. From these data, the scaling relation between dimensions and absolute molecular mass was obtained with one sample of each polymer. The molecular mass (\(M_r\)) of the complexes of monoclonal anti-bovine serum albumin
(BSA) and monomer BSA were determined on-line [115] by means of SEC, coupled with a low-angle laser light-scattering (LALLS) detector and two concentration detectors, ultraviolet (UV) and refractive index (RI) (SEC/LALLS/UV/RI system). Also, the size and \( M_r \) of the complexes were evaluated by the SEC/LALLS/UV/viscometer system. The principle of the differential viscometer detector and its use in constructing a universal calibration were explained by Titterton [116]. The pioneers in the field of viscometry detection in SEC were Fishman and co-workers [117], who first reported this technique in 1989 in studies of pectins. The use of SEC with on-line light-scattering, absorbance, and RI detectors for studying proteins and their interactions, was the topic of a review by Wen et al. [118]. Aqueous SEC with on-line argon-ion MALLS photometry and differential viscometry detectors was reported by Muller and co-workers [119]. Starch was characterized by HPSEC with detection by both MALLS and RI [120].

HPSEC with UV absorbance and on-line dissolved-organic-carbon (DOC) detectors have also been adopted and optimized under various conditions [121]. For example, an enhanced HPSEC/UV system with a modified DOC detector provides an improved understanding of the qualitative and quantitative natural-organic-matter (NOM) properties in water samples by detecting aromatic and non-aromatic fractions of NOM as a function of molecular weight. The “retention analysis method”, which is based on SEC in conjunction with an arsenic-specific detector (graphite furnace atomic-absorption spectrometer) was reported by Gailer and Lindner [122].

An interesting approach to on-line combination of SEC and GC has been designed, using LC/GC apparatus. This has been applied to determine organophosphorus pesticides in olive oil [123]. Per-O-sulfonated polysaccharides, including glycosaminoglycans and hyaluronan oligosaccharides have been analyzed by HPSEC with suppressed-conductivity detection [124]. The sensitivity of this method was compared to that of HPSEC with UV or fluorescence detection after reaction with 2-cyanoacetamide in strongly alkaline solution. The use of conductivity detection without derivatization and under isocratic conditions gave a limit of detection in the picogram range. Such a detection system is desirable for HPSEC of all polyelectrolytes.

HPSEC, combined with multispectral detection by a photodiode-array (PDA) UV detector was applied to the analysis of proteins and peptides in human cerebrospinal fluid (CSF) [125] and to the characterization of proteinergic profiles in the CSF of alcoholics [126]. Molecular components of the CSF were identified, and their purity was tested. The PDA detector, recently reviewed by several authors [127–133], provides significant savings of biological samples, as the entire spectrum can be registered simultaneously in a single run. Moreover, this technique eliminates possible errors caused by, e.g., a shift in retention time, which may occur when manual injectors are used. Several papers were published on the identification of various compounds and on purity tests performed with this type of detectors [134–137]. The application of multi-wavelength detection in the study of unfolding equilibrium of growth hormones in urea by SEC has also been investigated [138]. On-line PDA instrumentation and comparison of spectral ratios figured in monitoring tertiary and quaternary structural changes associated with protein denaturation. Stationary-phase-induced effects on protein conformation were monitored by changes in the maximum-to-minimum ratio of the second-derivative spectrum. Commercial protein preparations were analyzed for purity on a size-exclusion column,
coupled to a PDA detector [139]. Dupont [140] studied degradation of gelatin in paper upon aging by means of aqueous SEC and UV-PDA detection. Spectroscopic characterization by PDA detection of urinary and amniotic-fluid proteins, fractionated by anion-exchange and SEC/HPLC, was described by Calero et al. [141]. A similar technique was applied by de Vries [142] for the analysis of heparins. Moreover, a rapid quantitative determination of aromatic groups in lubricant oils by SEC combined with PDA detection was described by Varotsis and Pasadakis [143].

5.5.1 Mass spectrometry

As a growing number of applications and developments of multi-dimensional LC separations for proteomics are expected in the future, combinations of various modes of LC (including SEC) and MS are currently of widespread interest. Two-dimensional LC (2D-LC) is now being applied more and more in high-resolution separations. This approach has been used mainly in proteomics studies, where a large number of components must be separated, and as a technique complementary to 2D-electrophoresis. Due to technical difficulties, 2D-electrophoresis still cannot achieve the reproducibility required for unambiguous comparison of two independent separations. Many combinations of 2D-LC have been investigated and their applicability has been demonstrated. One of these approaches utilizes SEC, e.g., in the separation of polyethylene glycols and surfactants [144]. Other combinations have been applied in the separation of peptides [145,146]. Interfacing LC techniques with MS is a rapidly growing strategy in analytical sciences (Chap. 10) [147]. However, some precautions must be taken before linking these two techniques in order to make them fully compatible with respect to the mobile-phase composition, flow-rates, size of molecules, scanning time, etc. Sandra and co-workers [148] and Lemiere [149] have provided some very useful practical tips. The instrumental set-up for nucleic acid analysis has been reviewed by Huber and Oberacher [150], for clinical and forensic toxicology by Marquet [151], for drugs of abuse by Moeller and Kraemer [152], for analysis and screening of combinatorial libraries by Shin and Van Breemen [153], for proteomics by Peng and Gygi [154], for metabolite identification in drug discovery by Clarke et al. [155], for general HPLC/MS by Erickson [156] and Niessen [157], for high-throughput quantitative analysis of biological material by Jemal [158], for pharmaceutical analysis by Ermer and Vogel [159], and in nanotechnologies by Guetens et al. [160]. Complex SEC assays for screening combinatorial libraries were reported by Schurdak et al. [161]. This list is a selection from only the recent literature, describing the application of various chromatographic techniques linked to mass spectrometers equipped with various ionization sources.

Capillary and nano-chromatography columns, such as capillaries for electrophoresis, produce separations within minutes. The major problem in fast separations stems from the relationship between peak-width and scan duration. If the scan speed is too slow, some components might be overlooked in the mass spectrum. Moreover, an MS/MS (or MSⁿ) experiment, even if performed automatically, requires several seconds for completion. A compromise between separation quality and the limitations of the mass spectrometer can be achieved by using a peak-parking method [162,163]. This technique substantially prolongs the signal in a mass spectrometer and also enhances its sensitivity without
significantly affecting the resolution. As the peaks are retained longer on the column by decreasing the flow-rate of the mobile phase, the retention time cannot be considered as constant. Post-run data obtained with ESI-MS, coupled to the chromatographic system, require a deconvolution procedure to reveal the actual masses of the detected components. Often, the presence of complex mixtures of substances and/or the presence of contaminants may affect this algorithm, leading to false mass assignments [105]. Therefore, pre-separation of molecules according to their size may contribute to the unequivocal identification of all ions.

A method has been developed for on-line pseudo-cell SEC/MS (PsC/SEC/MS), providing rapid, real-time analyses of non-covalently bound protein complexes [164]. The methodology can be used to determine components of such complexes, as well as their exact stoichiometry. Furthermore, it enables the efficient determination of gross conformational changes upon complexation. The power of this new approach was demonstrated in the analysis of the global transition-state regulator AbrB and its complex with a target DNA sequence from the promoter sinIR. Non-covalent interactions were studied with the aid of SEC/coordinated ion spray (CIS)-MS [165]. Characterization of non-covalent complexes of antigen and recombinant human monoclonal antibody by cation exchange, SEC, and Biacore (detection and monitoring of the binding of biological molecules by surface-plasmon resonance technology) was reported by Santora et al. [166]. The effect of enzyme inhibitors on protein quaternary structure, determined by on-line SEC/micro-ESI/MS, has been described [167]. Blom et al. [168] have reported the determination of affinity-selected ligands and of binding affinities by on-line SEC/LC/MS. The use of SEC, linked to ESI-MS for the separation of neuropeptides and their fragments has been the subject of several papers [105,169–173]. A SMART™ System for micropurification was applied, using a Superdex Peptide column of 3.1 mm ID. Combination of such a system with MS required a volatile buffer, acceptable by the ESI source. In this case, a mobile phase consisting of 0.1% aq. TFA was found optimal. TFA generally suppresses the MS signal and decreases the sensitivity of measurements. Dilute formic acid gives much better results in this respect, but it was difficult to elute components from the column using this solvent. Capillary columns of Superdex Peptide have been applied for the identification of peptides and proteins by LC/MS [174]. Further details of this method have been described by Suder et al. [175]. An off-line identification of endogenous LVV-hemorphin-7 from CSF was performed on the Superdex Peptide column, followed by ESI-MS analysis of collected fractions [176].

Characterization of arsenic species in clams by multi-dimensional LC, linked to ICP-MS and ESI-TOF-MS/MS has been described by McSheehy et al. [177]. Such combinations of ICP-MS with LC have been receiving increasing attention in recent years. ICP-MS can also be applied to the detection of labile biological molecules, such as DNA fragments, where phosphorus can be monitored. Fractionation of phosphorus and trace elements in soybean flour and bean seeds by SEC, linked to ICP-MS was reported by Koplik et al. [178]. This group also discussed the application of various SEC columns to the separation of several other elements. Metal distribution patterns in cytosols from the mussel Mytilus edulis were demonstrated by Ferrarello et al. [179], who used SEC and double-focusing ICP-MS detection. Quantitative analysis of iron speciation in meat by using a combination of spectrophotometric methods and HPLC, coupled to sector
ICP-MS, has also been reported [180]. Wang and co-workers [181] have described the determination of trace elements in liver proteins by SEC/ICP-MS with a magnetic-sector mass spectrometer. A multi-dimensional approach, combining LC with parallel ICP-MS and ESI-MS/MS for the characterization of arsenic species in algae was reported by McSheehy et al. [182]. The identities of all the species were doubly checked, by matching the retention times of chromatographically pure species with standards and by ESI-MS/MS. The same group applied a similar approach in their investigation of arsenic speciation in oyster test reference material by multi-dimensional HPLC/ICP-MS and ESI-MS/MS [183].

Liu et al. [184] have recently reviewed the multi-dimensional separations of proteins and peptides. Micro-SEC on MicroSpin Sephadex G-25 columns was applied in combination with capillary LC/atmospheric-pressure chemical-ionization (APCI)-MS and used for the screening of potential drugs in recently discovered pharmaceutical compounds [185]. Harris et al. [186] have developed a size-exclusion-based system for the rapid isolation of plasmid DNA in a 96-well microplate format. A method for analyzing polysaccharide materials has been described, which employs SEC, followed by detection by on-line ESI-MS and off-line MALDI-TOF-MS [187]. The well-established method of two-dimensional gel electrophoresis is far too slow for screening in proteomics [188]. A new methodology has been developed, combining capillary electrophoresis/isoelectric focusing (IEF) with MS with sequential fragmentation (CIEF/MSn), and preparative IEF followed by SEC, combined with MS. Isotope ratio mass spectrometry was used to detect very low alterations in 13C abundance in analyte species that cannot be volatilized [189]. Examples were given of proteins, carbohydrates, and nucleotides, eluted from various types of HPLC columns.

Recent developments in MALDI-TOF techniques have enhanced the opportunities of linking HPLC with this ionization method. The MALDI source differs from the ESI source in that it operates in high vacuum. The high-vacuum source is capable of accommodating the LC mobile phase at a maximum flow-rate of 5–7 μL/min. Introduction of a liquid at higher flow-rates would cause an immediate shutdown of the instrument. Two different approaches to the on-line linking of SEC to the MALDI-TOF-MS, continuous-flow and aerosol, were discussed by Fei and Murray [190]. Details on linking capillary SEC to MALDI-TOF-MS can be found, e.g., in Ref. 193. In the aerosol method, the sample eluted from the column is mixed with matrix before nebulization. Typically, flow-rates are maintained between 0.5 and 1.0 mL/min. The problems with this type of interface have recently been solved by the use of a rotating-ball inlet [191], which prevents clogging of the vacuum interface by matrix crystals or frozen solvents. An alternative strategy utilizes a picoliter sampling onto the MALDI target plate with the help of a flow-through piezoelectric micro-dispenser [192], but larger robotic systems can be connected as well. Another promising methodology is based on the development of a MALDI source, operating at atmospheric pressure. Such a device can be mounted instead of an ESI sprayer and coupled to any of the known detectors (e.g. ion-trap, TOF, quadrupole). Promising developments in atmospheric-pressure MALDI (AP-MALDI) [193–196] and the application of an infrared laser at 3 μm may be a turning point for on-line LC/MALDI-MSn applications. The additional advantage of the IR laser is that ions can be generated from water solutions. This makes the system compatible with the LC separations of
biological molecules. On the other hand, Blais et al. [197] have discussed some limitations of the MALDI-TOF method in comparison with SEC for the characterization of phosphorus-containing dendrimers.

5.5.2 Nuclear magnetic resonance and surface-enhanced Raman spectroscopy

Another approach to unambiguous identification in LC is linking the chromatographic system with other structurally specific methods, such as NMR or surface-enhanced Raman scattering (SERS) [198–201]. The feasibility of interfacing flow-injection-based SERS methods with HPLC for the detection of individual components in a complex mixture was proposed in 1990 [202], but this approach has been studied more extensively in conjunction with capillary electrophoresis rather than with liquid chromatography. A microcoil NMR probe for coupling microscale HPLC with on-line NMR spectroscopy was described by Subramanian et al. [203] and adapted to the nanoliter scale by Behnke et al. [204]. These methods were developed for reversed-phase columns, but the same approach can also be applied to SEC. Application of LC, coupled on-line to $^1$H-NMR, was reported for the investigation of flavonoids [205]. The eluent was a mixture of MeOH and D$_2$O. Separation and identification of terpenoids were achieved by coupling a commercial capillary HPLC system with a PDA detector and a custom-built NMR flow microprobe [206]. The eluent from a 3-$\mu$m-diameter C$_{18}$ HPLC column was linked to a 500-MHz $^1$H-NMR microcoil probe with a volume of 1.1 $\mu$L. Direct on-line LC/NMR/MS/MS for the rapid screening of natural products was described by Sandvoss et al. [207]. An off-line MS/NMR procedure has been developed for rapid screening of small organic molecules and their ability to bind a target protein [208]. With this methodology it was also possible to obtain structure-related information as part of a structure-based drug discovery-and-design program. The methodology combines the inherent strengths of SEC, MS, and NMR to identify bound complexes in a relatively universal high-throughput screening approach. Another indirect technique was presented by Venter et al. [209] for the identification of membrane-transport proteins of E. coli. With a number of polymer additives as model compounds, practical problems encountered with multiple combinations were described for the coupling of HPLC with UV detection, on-line NMR spectroscopy, and MS, combined with a dedicated interface to collect chromatographic eluents for subsequent FT-IR [210]. SEC was performed with deuterated chloroform as eluent, the separation being monitored on-line by UV detection at 254 nm and on-flow $^1$H-NMR and MS. Interfacing LC with NMR and other detection techniques has recently been reviewed by Wilson [211]. A separate chapter of his review was devoted to SEC/NMR/IR instrumentation. A fully integrated system, involving HPLC, with superheated D$_2$O as a mobile phase, and combined with on-line PDA-UV, $^1$H NMR, FT-IR, and APCI-MS has been applied for the analysis of ecdysteroid-containing plant extracts [212]. The potential of such “multiple hyphenation” can yield a complete and unambiguous structural elucidation from a single experiment. The methods and theoretical basis for quantitative measurements in continuous-flow HPLC/NMR were presented by Godejohann et al. [213].
5.6 CALIBRATION

Deviations from the Benoit principle of universal calibration were observed by Belenkii et al. [214] when flexible-chain polymers were chromatographed on macro-porous swelling sorbents. These peculiarities were caused by different degrees of thermodynamic compatibility of the polymers with the sorbent matrix. More recently, a new SEC method for the estimation of the weight-to-number-average molecular-weight ratio $M_w/M_n$ of polymers with a narrow molecular-weight distribution, approximated by a log-normal distribution, was proposed [215]. The method was applied to a series of polystyrene standards of narrow molecular-weight distribution. Guillaume et al. [216] have proposed a mathematical model for hydrodynamic and SEC of polymers on porous particles. The model described constitutes an attractive method for enhancing these two chromatographic techniques for separating biological or synthetic macromolecules. Different polynomial models of calibration curves have been evaluated and compared with respect to their predictive properties [217]. The best model across the effective fractionation range (linear range) was not always found to give a straight line. Polycyclic aromatic hydrocarbon standards were applied in studies on the polymerization of anthracene oil with $\text{AlCl}_3$ as the catalyst [218]. SEC separations have been carried out on a stationary phase of polystyrene/polydivinylbenzene with 1-methyl-2-pyrrolidinone at 80°C as eluent. Endogenous calibrants were used by Tsao et al. [219] for the quantitation of various molecular forms of chromogranin A in serum and urine. Oliva et al. [220] performed a comparative study of protein molecular weights by SEC and laser-light scattering. The results obtained by the two methods were compared, using samples of recombinant human growth hormone and $\beta$-lactoglobulin as test substances. The effect of peak-broadening and of the error in inter-detector volume on the calibration curve and experimental molecular-mass averages obtained by SEC were investigated by Netopilik [221]. The parameters affecting the fractionation performance in SEC of broad polymer samples were studied by Lou et al. [222]. Two different modes were considered, i.e., using MALDI-MS to provide an absolute calibration curve for SEC, and using SEC as a sample preparation step for MALDI-MS measurements. The latter combination was demonstrated to be more reliable, because most problems inherent in SEC can be circumvented.

The pore dimensions, pore-size distributions, and phase ratios were determined [223] for a set of cation exchangers, using inverse SEC (ISEC). This technique [224] is alternatively called macromolecular porosimetry [225] and is used for the characterization of the porosity of materials [226]. The adsorbents examined represent a diverse set of materials from Pharmacia, Tosohas, BioSepra, and EM Industries. The ISEC was carried out using dextran standards. This technique provided a comparative characterization of the accessible internal pore-surface area, as a function of solute size. ISEC was adopted to measure the permeability of microcapsules (hollow hydrogel spheres with diameter $<1$ mm), using dextran molecular-weight standards [227]. Alginate/poly(1-lysine)/alginate microcapsules were chosen as a column substrate. Polysaccharides of known molecular weight were used as standards for HPSEC of humic substances [228]. Calibration curves were equivalent for both columns, whereas analytical parameters revealed that a TSK column was only slightly more efficient in separating polysaccharide
standards. Variations between columns in the results obtained for the molar masses of humic substances were attributed to intrinsic properties of these substances, such as stability of conformational structures.

A universal calibrator has been applied to the determination of molecular masses of heparin samples and has been referred to as the Heparin Molecular Mass Calibrant [229]. This calibrator replaces the large number of calibrators (19 in the authors’ previous work) that are required for molecular-mass analyses. Polysaccharide standards having average molar masses in the range of 180 – 100,000 Da were used in HPSEC, applied as a screening technique for the determination of average molar masses of polygalacturonic acid samples for use in pharmaceutical applications [230]. The method was employed in screening commercially available polygalacturonic acid raw materials with respect to both average molar mass and polydispersity. The analysis of poly(bisphenol A carbonate) by SEC/MALDI was reported by Puglisi et al. [231]. Their results show that MALDI spectra of the SEC fractions allow not only the detection of linear, and cyclic oligomers, but also the simultaneous determination of their average molar masses. Two slightly differing SEC calibration plots were obtained, due to the smaller hydrodynamic volume of the polycarbonate cyclic chains relative to the linear ones. The possibility of standardizing calibrants for SEC was investigated by Yomota and Okada [232]. Their review article (unfortunately in Japanese only) focuses on the water-soluble polysaccharides, such as dextran, hyaluronate, and chitosan. A HPSEC/RI method has been described for the quantification and molecular-weight determination of extractable water-soluble polyamines in a novel, proprietary, polymeric pharmaceutical compound [233]. The extracted polyamines were synthetic impurities as well as potential degradation products of the polymer. Potzschke et al. [234] studied the molar masses and structure of hemoglobin hyperpolymers, commonly used in calibrations for SEC of these artificial oxygen carriers. The calibration curve was found to differ significantly from that given by native globular proteins due to a less compact structure of hemoglobin hyperpolymers. Therefore, the calibration of SEC with globular proteins for the determination of molar masses of hemoglobin polymers would be erroneous.

A method for the calibration of SEC columns, suggested by Harlan et al. [235], takes into account the nonlinear dependence of the Stokes radius, $R_s$, upon the partition coefficient, $K_D$. An application of this method, in which aggregation states of the membrane protein prostaglandin H2 synthase, solubilized in nonionic detergents, was reported by Duggleby [236]. He described a method and software with appropriate statistical operations to select the best straight or curved calibration line. The size of the unknown is then interpolated and an estimation of the error is calculated. Le Maire et al. [237] tested the hypothesis that porous media used in protein SEC are surface fractals. The data obtained in the calibration of “classical” gels (Sephacryl and Sepharose) and of HPLC phases (TSK SW and PW) with proteins having a wide range of molecular sizes were analyzed within the framework of this theory. While the model does not apply to “classical” gels, it seems that HPLC phases can be described as fractals over the range of protein sizes. The same group [238] also tested the hypothesis [239] that the elution position of macromolecules in gel chromatography is better correlated with the viscosity-based Stokes radius ($R_\eta$), rather than with the Stokes radius ($R_s$) calculated from the frictional coefficient. By the use of different gel matrices (agarose, Sephadex, and TSK
silica gel columns) it was found that the elution positions of dextran fractions and reduced proteins, denatured with guanidine hydrochloride, were in accordance with their $R_n$. In this case, water-soluble, globular proteins were used for gel calibration. A simple routine for nonlinear least-square analysis was described [240] and applied to small-zone scanning data, where calibration of the gel column requires the use of fully characterized markers of known molecular size. Application of nonlinear least-squares analysis eliminates the difficulty encountered due to the scarcity of calibrating markers for gels whose porosities span a certain size range. A multilevel calibration has been discussed by Hinshaw [241]. By misapplying multilevel nonlinear calibration to compensate for gross systematic errors, one can mask serious malfunction of the analysis, leading to erroneous results. The author concludes that “it’s never a good idea to assume that a chromatograph delivers equal responses to all components or at all levels”. This is obviously true and should always be considered, not only with regard to chromatographic data, but also in MS and other techniques. An overview of the chemometric methods for calibration, optimization, and statistics can also be found in Ref. 242. Lazaro et al. [243] investigated the elution behavior of coal-derived materials on a polystyrene/divinylbenzene SEC column with 1-methyl-2-pyrrolidinone as the mobile phase and use of polystyrene calibration. This calibration was also correlated by mass spectrometry. The importance of calibration was emphasized in the measurement of dextran clearance in clinical studies [244].

5.7 APPLICATIONS

5.7.1 Polymers

Determination of molecular weight and molecular-weight distribution is crucial for understanding the relationship between the properties of polymeric materials and their molecular structures. The molecular-weight averages and distributions are given by the principal relationships defined in terms of number-average molecular weight ($M_n$), weight-average molecular weight ($M_w$) and $z$-average molecular weight ($M_z$). Further parameters to be considered are the $(z + 1)$-average molecular weight as well as the viscosity-average molecular weight ($M_\eta$). These averages are all connected with the population defined by the number of moles ($n_i$), or weight ($w_i$) of individual macromolecules possessing molecular weight $M_i$, contained in the polymer population. The distribution of polymer molecular weight is described by various mathematical relations, including Gaussian, Poisson, Flory-Schulz, and log-normal molecular weight distribution functions. The polydispersity of polymers, $Q$, is traditionally defined as the relation of their weight-average to number-average molecular weights

$$Q = \frac{M_w}{M_n} = U + 1$$  \hspace{1cm} (5.4)

where $U$, the molecular inhomogeneity, has a numerical value of one less than $Q$. Polymers are usually distributed in more than one direction of inhomogeneity, which comprises, in addition to molecular-mass distribution (MWD), the distribution with respect to chemical composition (CCD), functionality (FTD) and topology (MAD). The chemical composition of polymers is related to the macromolecular chain structure,
including the number of co-monomer units for co-polymers, their sequence of incorporation, configuration, and conformation. The topology of synthetic polymers concerns the polymer-chain architecture (linear, comb-like, branched, cyclic, etc.), and is connected with functionality, *i.e.*, with the number and localization of the functional groups.

Several approaches have been used for the separation of polymers according to both MWD and CCD. In contrast to homopolymers, which are monodisperse with respect to CCD for each retention volume, different combinations of molar masses, composition, and sequence length can be found in co-polymer fractions having the same hydrodynamic volume. In order to address this problem, several selective detection techniques have been combined with SEC. Hyphenated techniques in SEC have been reviewed recently [245]. Two-dimensional chromatography is another approach frequently used for the characterization of molar mass, composition, and functionality distributions of polymer macromolecules. The hyphenation of SEC in a two-dimensional “orthogonal” mode has been applied to the characterization of amphiphilic copolymers [246–248]. Full co-polymer characterization can be achieved by measuring the bivariate distribution, *i.e.*, with respect to masses and composition. This measurement is particularly important for co-polymers obtained at high conversion of co-monomers, because, usually, at that stage of co-polymerization, one of the two monomers is preferentially incorporated into the co-polymer chain. Therefore, the spread in composition increases as conversion progresses, and the resulting co-polymer displays compositional heterogeneity. Several methods have been suggested to determine the bivariate distribution of masses and composition in co-polymers. For these purposes, the co-polymer is usually fractionated by SEC, and the collected fractions are subsequently analyzed by various spectrometric techniques [249]. For example, the fractions can be analyzed by NMR to determine the co-polymer abundance and composition and then by MALDI-MS to determine the molar mass of each fraction. Finally, NMR and MALDI-MS data are combined with bivariate distribution models to yield bivariate distribution maps [249].

The molecular size of a homo/co-polymer molecule in solution is a function of its chain length and chemical composition, solvent, and temperature. It is, therefore possible to selectively separate polymers with respect to hydrodynamic volume, chemical composition, and functionality by the use of different modes of LC. When the adsorption effects between the polymer molecule and the column packing particles are dominated by the enthalpic interactions, $K_D > 1$ in the SEC general retention equation (Eqn. 5.1), and chromatography takes place in the adsorption mode. It is generally assumed that adsorption chromatography is more suitable than SEC for the characterization of chemically heterogeneous polymers. This can be performed in either isocratic or gradient elution modes. Gradient polymer elution chromatography (GPEC) in both reversed-phase (RP) and normal-phase (NP) modes have been applied in microstructural characterization of aromatic co-polymers [250]. Several examples of GPEC and its combination with SEC for the determination of CCD of co-polymers as well as FTD of telechelic polymers, polymer blends, and resins have been reported [251]. Determination of polymer structure by SEC is, besides protein research, one of the largest field of applications. SEC is capable of providing both, the molecular mass distribution and the polydispersity index. On-line linking with ESI-MS or combination with MALDI-TOF-MS as specific detectors provides
full structural information, including data on the nature of the end groups. Several reviews describe the application of SEC in the field of synthetic polymers [252].

A strategy for controlling the polymer topology through transition-metal catalysis has been described by Guan [253]. The molecular weight and intrinsic viscosity of the hyperbranched polymers were measured by MALDI-MS and SEC with triple detectors. Characterization of polyether and polyester polyurethane soft blocks was performed by MALDI-MS and compared with SEC/MALDI [254]. The SEC/MALDI results provide significantly larger values of $M_w$ and polydispersity than MALDI alone. SEC is now considered to be a standard tool in the molecular characterization of commercial polymers. In this respect, two factors, i.e. repeatability and reproducibility, are important. In order to determine the variance in reproducibility, several interlaboratory studies (i.e., round-robin experiments) have been undertaken, and the round-robin experiments conducted in Europe have done much to raise awareness of quality issues in SEC.

Recently, the results of an interlaboratory experiment in high-temperature SEC have been published [255]. Fifteen laboratories performed analyses of five polyethylene samples and two standards. It was found that the reproducibility, measured by the interlaboratory standard deviation ($s_{LAB}$) was greatly influenced by the width of the molecular-weight distribution (MWD) and branching. The $s_{LAB}$ values for $M_w$ of linear polyethylenes of narrow and broad MWD were 4 and 14%, respectively. For branched polymers, SEC/viscometry methods were shown to measure significantly higher molecular weights than the non-hyphenated SEC method. For single-site polyethylene, only a couple of laboratories reported the MWD that closely matched the Flory distribution. It was concluded that many variations in instruments and analytical methods exist among laboratories, and that this technique must yet undergo many refinements before a truly standard method is widely accepted and implemented. Nevertheless, wide-ranging applications of SEC to the characterization of synthetic polymers, resins, coatings and paints were demonstrated, and some of the recent examples are given below. Calculations of the $\eta/M$ relationship for ethylene/propylene co-polymers have been performed, and the results were used for a theoretical examination of the effect of co-polymer composition on the calibration of SEC columns [256]. The kinetics of peroxide-induced degradation of polypropylene has been investigated by comparing the molecular-weight distributions, as determined by SEC measurement, and the impact of imperfect mixing on the MWD shift has been examined [257].

The application of SEC to the quantitative measurement of adhesion between polypropylene blends and paints, self-nucleation behavior of the polyethylene block in polystyrene/β-polyethylene/β-polycaprolactone triblock co-polymers, characterization of the molecular structure of highly isotactic polypropylene, as well as the use of SEC for the determination of chain-scission distribution function for polypropylene degradation, constitute some further examples of the use of this technique in studies of polyolefins [258–261]. Polypropylene oligomers were isolated from the polymer matrix and have been characterized by a combination of SEC with FTIR spectrometry and HPLC with UV detection [262]. An analytical strategy recently developed [263] for the analysis of polyisobutenylenes, partially functionalized with isothiocyanate groups, involved coupling of capillary SEC, SEC, and RP-LC. By comparing the results obtained with these techniques, a complete characterization of the polymer was achieved, and the degree of
polymerization and relative quantity of the different series of macromolecular chains were estimated. SEC has been further utilized for the characterization of isobutylene co-polymerizations of various topology, such as poly(isobutylene/β-styrene) block co-polymer, triblock co-polymer with densely grafted styrenic end blocks (prepared from a poly-isobutylene macro-initiator), novel multiarm-star polyisobutylene/polystyrene thermoplastic elastomers, linear and star-shaped block co-polymer of isobutylene, methacrylates (obtained by combination of living cationic and anionic polymerizations), and multiarm-star polyisobutylenes [264–268]. The application of SEC to PVC (polyvinyl chloride) characterization includes the determination of the MWD of commercial PVC, and was recently reported [269–271].

A comparison of the SEC performance with that of size-exclusion electrochromatography (SEEC) for the mass distribution analysis of synthetic polymers, such as poly(methylmethacrylate) (PMMA), polycarbonate, polycaprolactam, poly(ethylene terephthalate), and polystyrene (PS) was recently published [272]. The repeatability of electro-osmotic flow control within-day, day-to-day and column-to-column was determined for SEEC with respect to retention and separation efficiency. It was shown that by using the retention ratio instead of the migration time, the precision of the mass distribution was sufficiently high, and that similar distributions were obtained for a sample analyzed by pressure-driven SEC and by SEEC. With the aid of direct on-line coupling of SEC with depolarization (D) multi-angle light scattering (SEC/DMALS), a method for studying optical anisotropy of polymers as a function of their molar mass has been developed [273]. The effects of tactic heavy-atom substitution on the main chain in the depolarization behavior of polymers were studied, using atactic and isotactic PMMA, atactic and brominated PS, and the semi-flexible polypeptide. An introduction to the theory of SEC/DMALS was also given. By combining gradient HPLC and SEC in a fully automated 2D chromatography setup, it was possible to simultaneously fingerprint the chemical composition and molar mass during investigations of the grafting of methylmethacrylate onto ethene/propene/diene rubber [274]. Preparative SEC was used for fractionation of highly branched PMMA with an estimated degree of branching (3.7 branch-points per 100 monomer units). The fractions were characterized in solution by SEC/viscosity coupling and in the melt by visco-elastic spectroscopy [275].

Two experimental techniques, namely, SEC with fluorescence detection and SEC, coupled to forward recoil spectrometry (FRES), were used to monitor independently the extent of reaction between model end-functional polymers at a PS/PMMA interface [276]. This is of importance for investigations of polymer/polymer reaction kinetics and interfacial segregation during in situ reactive polymer compatibilization. Possibilities and limitations of photon correlation spectroscopy (PCS) in determining polymer molecular-weight distributions were studied in comparison with the results obtained by SEC for samples of PMMA having monomodal and bimodal distribution functions [277]. Evaluation of a single-capillary viscometer detector, coupled on-line to a SEC system, was performed, using various polymers that were soluble in organic (PS, PMMA, polyvinyl acetate (PVAc), PVC, polyalkylthiophene), and aqueous solvents (PEO, PEG, pullulan, and hyaluronan). Molar-mass distribution, intrinsic-viscosity distribution, and constants of the Mark-Houwink-Sakurada relationship were determined [278].
Using polymers of complex macromolecular architectures, Stogiou et al. [279] recently tested the validity of the universal calibration curve (UCC) in SEC. The polymers studied included the microarm stars, H-shaped, and pi-shaped, as well as a model linear tetrablock co-polymer of the PS/PI/PS/polyisoprene type. It was found that the universality of the relation of log M\(\eta\) vs. peak elution volume is also valid for these complex molecules. However, the determination of the molecular weight of a polymer with the UCC was found to be very sensitive to the molecular and compositional homogeneity of the sample. A qualitative analysis of secondary mechanisms in SEC of polymers through the mean value of the viscosimetric exponent has been reported by Gomez et al. [280]. A computer simulation study for estimating the biases induced by branching under ideal fractionation and detection conditions was performed for the analysis of a styrene/butadiene graft co-polymer by SEC [281,282]. A novel polymerization model was developed for predicting the MWD, DBD (degree-of-branching distribution), and CCD for the total co-polymer and for each of its different branched topologies. To simulate the molecular-weight calibrations in SEC, the Zimm-Stockmayer equation was applied to each co-polymer topology. Negligible deviations due to branching were found in the MWD and the DBD with respect to the theoretical predictions. However, errors in the CCD were intolerably large and, consequently, it was concluded that the CCD cannot be estimated by SEC.

Park et al. [283] have recently investigated the reaction products of polystyryllithium with air by SEC, temperature-gradient interaction chromatography (TGIC) and MALDI-TOF-MS. It was confirmed that polystyryl ketone, polystyryl alcohol, and directly coupled polystyrene were the major products, in addition to the normally terminated polystyrene. Moreover, polystyrenes, end-capped with methoxy and carboxylic acid groups, as well as dipolystyryl ether, were also identified as minor products. Comparative studies on polymer characterization by TGIC and by SEC have been reported by Chang et al. [284]. TGIC is a form of HPLC in which the column temperature is changed in a programmed manner to control the retention of polymeric species during isocratic elution. The polymers were separated by TGIC in terms of their molecular weights. TGIC was considered to be superior to SEC with respect to resolution and sample loading capacity, and was found to possess higher sensitivity to molecular weight in the analysis of nonlinear polymers. The advantage of TGIC over solvent-gradient HPLC arises from the fact that the former permits the use of RI detection methods, such as differential refractometry and light scattering due to the use of isocratic elution. In addition, temperature variation provides finer and more reproducible values of retention volume than does variation in solvent composition. Such control is important when the MWD is determined by secondary calibration methods. TGIC was successfully applied to the characterization of star-shaped polystyrene, and kinetics of linking between living polystyrene anions and chlorosilane linking agent were investigated in detail. Thermodynamic principles and some applications of the TGIC analysis were reported, and it was found that the MWD of anionically polymerized species is much narrower than generally assumed from SEC analysis. The TGIC separation conditions for PS, PI, PMMA, PVC, and PVAc over a wide molecular-weight range were established. Studies of band-broadening occurring in SEC were performed, using very narrow PS standards, obtained and characterized by TGIC. Recently, TGIC was applied in the separation of stereo-regular polyethyl methacrylate (PEMA) according to the polymer tacticity. To isolate the tacticity effect from the
molecular-weight effect on TGIC retention, the PEMA samples were fractionated by TGIC, and the accurate molecular weights of the fractions were determined by MALDI-TOF-MS. It was concluded that the retention in TGIC was affected by both the tacticity and the molecular weight [285,286].

A review on the recent progress in combining a full adsorption/desorption procedure with SEC (FAD/SEC) to the separation and characterization of co-polymers has been published by Nguyen and Berek [287]. FAD includes complete and selective adsorption of the polymer sample to be separated from a solvent promoting adsorption onto an appropriate adsorbent, which is packed into a designed LC-type microcolumn. In the following steps, macromolecules were displaced from the adsorbent by different eluents with increasing desorbing strength. The fractionation of polymers according to their MWD and CCD can be accomplished in the course of the FAD process [287]. Another review, covering the application of SEC to lipophils and biopolymers was also published by Berek [288]. A theoretical model and data analysis methods were recently proposed for SEC of step-growth polymers with cyclic species [289]. The measurement of MWD of many step-growth polymers is complicated due to the presence of cyclic oligomers, formed during polymerization. When SEC is used to determine the MWD, the cyclic oligomers are generally only partly separated from the linear polymer and, hence, distort the measured linear MWD. Moreover, the cyclic oligomers require a different calibration curve, in contrast to the linear species. Therefore, in general, their molecular weights are not accurately measured. The proposed model of the SEC separation of step-growth polymers with cyclic species was used for the characterization of these polymers by conventional and multi-detector SEC. The results were compared with experimental data for nylon 6, nylon 6.6, and polyethylene terephthalate.

The molar mass distribution of several functional oligo-amides has been studied by SEC [290]. The molar masses determined by SEC were compared with the values obtained by chemical titration and by NMR analysis. The kinetics of the reaction of tosyl isocyanate with polyhexamethylene/pentamethylene carbonate diol has also been evaluated by SEC [291]. Studies on polybisphenol A carbonate (PC), by SEC/MALDI have been conducted by Montaudo and co-workers [292,293]. The investigations included end-group and molar-mass determination, as well as examination of self-association, and the mechanism of thermal oxidation. The results showed that MALDI spectra of the SEC fractions allow not only the detection of linear and cyclic oligomers in the polymer, but also the simultaneous determination of their average molar masses. Two slightly differing SEC calibration plots were obtained, due to the smaller hydrodynamic volume of the polycarbonate cyclic chains, compared to the linear ones. In agreement with theory, the \( (M_{\text{cycle}}/M_{\text{linear}}) \) ratio at a fixed elution volume was found to be 1.22, independent of the molar mass values. Thermal oxidation products of PC, generated by heating at 300 and 350°C in air, were detected by SEC/MALDI. The SEC curves of the thermally oxidized samples showed extensive degradation as a function of heating time, up to the formation of oligomers having very low masses. Oxidized PC samples were subjected to SEC fractionation with collection of several fractions that were further analyzed off-line by MALDI/TOF. The mechanisms accounting for the formation of thermal oxidation products of PC involved several simultaneous reactions:
(a) hydrolysis of carbonate groups of PC to form free bisphenol A end groups,
(b) oxidation of the isopropenyl groups of PC, and
(c) oxidative coupling of phenolic end-groups to form biphenyl groups. The presence of biphenyl units among the thermal oxidation products confirmed the occurrence of cross-linking processes, which are responsible for the formation of the insoluble gel fraction.

The biological polyesters have been systematically investigated [294], and SEC and ESI-MS have been used to determine the sequence distribution and chemical structure of mass-selected macromolecules of poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV), a natural polyester macro-initiator, obtained by partial alkaline depolymerization of natural PHBV [295]. The microstructure of this bacterial co-polyester was assessed, starting from the dimer up to the oligomer containing 22 repeat units, and the results obtained were compared with those described previously involving other “soft” ionization MS techniques. The poly-3-hydroxybutyrate (HB)/co-ε-caprolactone (CL) co-polymers, derived via acid-catalyzed trans-esterification of natural PHB have been characterized by SEC and MALDI-TOF [296] with regard to their molecular weights, molar compositions, and average block length of repeating units. The MALDI-TOF mass spectra of samples fractionated by SEC made it possible to ascertain that co-polymers rich in HB units had mostly hydroxyl and carboxyl end-groups, while copolymers rich in CL units had mostly tosyl- and carboxyl end groups. The structure of biomimetic PHB and poly-2-methyl-3-hydroxyoctanoate was established by ESI/MS and SEC analyses. The addition/removal mechanism of the polymerization of β-lactones containing α-hydrogen by alkoxide anion was demonstrated to be true for β-lactones having alkyl substituents in both α- and β-positions [297]. The comparison of SEC and ESI-MS results for the analysis of water-soluble racemic PHB oligomers (from dimer to dodecamer) was performed, based on ESI-selective ion-display patterns of individual macromolecular ions [298]. The structure of the water-soluble oligomers, composed of racemic PHB, covalently conjugated to l-alanine and Ala-Ala-Ala oligopeptide has also been assessed by ESI/MS, and the respective structural information on each has been complemented by SEC analyses [299]. SEC and MALDI-TOF-MS have recently been used to determine product molecular weight and end-group structure in mass-selective lipase-catalyzed poly(ε-caprolactone) transesterification reactions [300]. These studies have shown how enzymatic transesterification reactions can be further developed to provide oligomers with well-defined length and end-group structure.

Novel block co-polymers, based on 2-vinylpyridine (2VP) and ε-caprolactone (CL), which are expected to be useful pigment-dispersing agents for TiO₂ in, e.g., polyester powder coatings, have been characterized by SEC [301]. It was found that part of the living 2VP chains was deactivated immediately after addition of CL, which yielded bimodal MWD. A comparison of SEC with ESI-MS and with MALDI-TOF-MS for the characterization of polyester resins has been reported. A series of 17 polyester paint resins has been analyzed in order to compare the structural and molecular-weight data derived from each technique [302]. The optimization of sample preparation and laser power were found to be important factors in obtaining constant MWD by MALDI. However, the use of SEC for the fractionation of polyester samples prior to ESI or MALDI analysis showed that both techniques significantly underestimated the average molecular weights of the
polydisperse polyesters. Differences in the relative abundances of branched and cyclic species in ESI vs. MALDI mass spectra were also noted. It was concluded that it would be useful to employ both techniques to ensure complete characterization of complex polymer samples by SEC. A novel analytical method, called two-dimensional correlation gel permeation chromatography, has recently been introduced in a study of the octyltriethoxysilane sol/gel polymerization process [303]. This technique, based on the combination of 2D-correlation analysis and time-resolved SEC, is a useful method for studying polymerization processes. A combination of LC with ESI orthogonal acceleration time-of-flight (oaTOF) MS has been applied in on-line polymer analysis [304]. In one experimental setup, three different LC modes were interfaced with MS: SEC/MS, GPEC/MS, and LC-CAP/MS.

### 5.7.2 Proteins and polypeptides

One of the challenges of the last decade has been the identification of critical cellular markers of neurodegenerative diseases. Such markers include amyloid beta (Aβ) protein and its fragments, prion proteins, etc. Cells and tissues contain thousands of proteins (an average of ca. 10,000 proteins per cell). A complete analysis of the entire protein content, as well as their structures and interactions with other molecules, is an objective of an increasing importance, bearing in mind the long-term goal of developing novel drugs. Handling of such complex mixtures has been the subject of many reviews, and despite several methodological breakthroughs [3,305–309], a consensus on the most suitable and reproducible strategy has not been achieved. A polymerized form of recombinant mouse prion protein (mPrP) domain 23–231 [mPrP-(23–231)], designated mPrP-z, was recently generated [310] and isolated by RP-HPLC or size-exclusion HPLC. Transgenic mouse Aβ peptides were purified by sequential SEC and RP-LC, and subjected to amino acid sequencing and MS [311]. Analysis of the in vivo-derived Aβ polypeptides by on-line 2D-chromatography/MS was performed to detect Aβ 1–40 and Aβ 1–42 directly in cell lysates [312]. The method consisted of on-line SEC to provide initial separation of analytes from the sample (based on their molecular mass), coupled with sample concentration prior to analysis by microbore LC/MS.

The chromatographic system Äkta-Purifier 10, scaled up for preparative HPLC, was used for the purification of substance P (SP) endopeptidase activity in the ventral tegmental area (VTA) of the rat brain [313]. By use of this strategy, it was possible to achieve a purification factor of almost 7500, based on the specific activity. Righetti and Verzola [19] discussed the usefulness of present methodologies in the study of folding/unfolding/refolding of proteins in comparison with the capabilities of capillary zone electrophoresis. The effect of enzyme inhibitors on protein quaternary structure has been determined by on-line SEC/MS [314]. SEC and electrophoretic separation techniques have been used by Underberg et al. [315] to investigate the physical stability of peptides and proteins. These authors also presented an overview of various separation and detection techniques for peptides and proteins that have been used in stability research and biochemical analysis. Assembling of γ- with α-globin chains to form human fetal hemoglobin in vitro and in vivo was verified by SEC [316]. Analytical-scale SEC was
applied to the large-scale production of recombinant hepatitis B surface antigen from *Pichia pastoris* [317].

Direct interaction of a high-affinity complex between the bacterial outer membrane protein, FhuA, and the phage T5 protein, pb5, has been demonstrated by isolating a pb5/FhuA complex by SEC [318]. Kim and Park [319] used this method to test the aggregation stability of encapsulated recombinant human growth hormone. The behavior of gelatin in dilute aqueous solution has been studied with the objective of designing a nanoparticulate formulation by using SEC under various conditions of time, temperature, pH, and ethanol concentration [320]. Kinetics and thermodynamics of dimer formation and dissociation for a recombinant humanized monoclonal antibody against vascular endothelial growth factor were investigated as a function of pH, temperature, and ionic strength by SEC, using the concentration jump method [321]. The relevance of techniques, such as analytical ultracentrifugation, SEC, and MS in the structural investigation of detergent-solubilized membrane proteins was discussed by LeMaire et al. [322]. A simple chromatographic assay for Rab geranylgeranyl transferase has been developed [323]. The method involves separation of the reaction mixture on a Sephadex G-25 Superfine minicolumn.

The proteome determines the cellular phenotype, and the regulation of the entire inter- and intracellular network requires simultaneous monitoring [324–326]. Data from 2D-separation techniques, such as 2D-gas chromatography (GC × GC), liquid chromatography/liquid chromatography (LC × LC), and liquid chromatography/capillary electrophoresis (LC × CE) can be readily analyzed by various chemometric methods to increase the information content of chemical analysis [327]. Proteolysis of whole-cell extracts with application of immobilized-enzyme columns as a part of multi-dimensional chromatography, was investigated by Wang and Regnier [328]. The effectiveness of proteolysis was evaluated with extracts of *E. coli*, the extent of degradation being monitored by SEC. Hille et al. [329] proposed a combined technique, involving capillary isoelectric focusing coupled to mass spectrometry (CIEF/MS") and preparative IEF, followed by SEC linked to MS (PIEF/SEC/MS) to improve automation, speed, and precision of proteome analysis. Peptide mapping with combinations of SEC, RP-LC, and CE was reported by Stromqvist [330]. Recombinant extracellular superoxide dismutase was proteolytically degraded by trypsin, and the digest was fractionated by three different separation techniques, among them SEC. Zhang et al. [331] showed how several preparative steps were essential for obtaining information about modified human lens β-crystallins. The preparative techniques prior to MS included SEC, RP-LC, 2D-PAGE, in situ digestion of the proteins, and peptide trapping before the final LC/MS analysis. To understand the structural properties of buffalo growth hormone, an equilibrium denaturation with guanidine chloride was carried out and was monitored by UV spectroscopy, intrinsic fluorescence spectroscopy, far-UV circular dichroism, and SEC [332]. Native, unfolded/refolded frutalin and a distinct molecular form denoted “misfolded”, were separated on Superdex 75 [333]. On-line coupling of SEC with imaged capillary isoelectric focusing and a membrane interface for proteins separation have been described by Tragas and Pawliszyn [334]. The system is equivalent to 2D-PAGE, transferring the principle of 2D-separation to the capillary format.
5.7.3 Molecular biology

SEC and denaturing gel electrophoresis of a recombinant enzyme was applied [335] to characterize NADH-dependent methylenetetrahydrofolate reductase from higher plants and to prove that it exists as a dimer of ca. 66-kDa subunits. SEC analysis of adenovirus particles has also been reported [336]. DNA binding properties of basic helix-loop-helix fusion proteins of Tal and E47 have been investigated [337]. It has been concluded from the SEC studies that all mutant and fusion proteins are dimeric. Pacek et al. [338] studied DNA box sequences as the site for helicase delivery during plasmid RK2 replication initiation in *E. coli*, and Shirakawa et al. [339] investigated the targeting of high-mobility proteins in chromatin. Meyer and co-workers [340] observed a difference with respect to dimer formation between native PrP(C) and recombinant PrP prion proteins. Application of various strategies, including SEC, to the purification of tumor-specific immuno-therapeutics, obtained by recombinant DNA technology, was reviewed by Matthey et al. [341]. SEC was also applied in an automated one-step DNA sequencing technique [342], based on the nanoliter reaction volumes and capillary electrophoresis. The reaction products were purified by SEC, followed by an on-line injection of the DNA fragments into a capillary. Over 450 bases of DNA could be separated and identified by this technique. A review including descriptions of various separation techniques used in nucleic acid research was published by Takenaka and Kondo [343]. For large natural polymers, such as double-stranded DNA molecules or synthetic polymers, slalom chromatography (SC) or hydrodynamic chromatography (HDC) can be applied. The elution order in HDC is the same as in SEC. On the other hand, the observed elution order of double-stranded DNA molecules is the opposite of that expected for HDC or SEC [344]. The theory and validation for these methods have been described [345–347]. All three modes can be treated as complementary and linked in a global separation mechanism, utilizing a nonequilibrium chromatographic principle.

5.7.4 Carbohydrates

Methods for the preparation and characterization of hyaluronan oligosaccharides of defined length have been recently reported [348]. The preparations obtained by SEC were characterized by a combination of ESI-MS, MALDI-TOF-MS, and fluorophore-assisted carbohydrate electrophoresis. Another approach to the analysis of polysaccharide materials employs SEC [349], followed by detection by on-line ESI-MS and off-line MALDI-TOF-MS. It was demonstrated that formation of the multiply charged oligomers that bind up to five sodium cations permits the rapid analysis of polysaccharides with molecular masses in excess of 9 kDa. Isolation and purification of proteoglycans by SEC was the subject of a review by Fedarko [350]. Characterization of the molar masses of hemicelluloses from wood and pulps by SEC and MALDI-TOF-MS has been reported by Jacobs et al. [351]. Negative-ion (NI)-ESI-MS and SEC have been used to reveal structural heterogeneity in κ-carrageenan oligosaccharides [352]. HP-SEC, combined with isotope-ratio MS, was applied by Abramson et al. [353] to measure low levels of underivatized materials, such as proteins, carbohydrates, and nucleotides. This combination produces a device capable of measuring very low alterations in
\(^{13}\)C abundance from analyte species that cannot be volatilized. The occurrence of internally \((1 \rightarrow 5)\) -linked arabinofuranose and arabino pyranose residues in arabinogalactan side-chains from soybean pectic substances was investigated by Huisman et al. [354], and the compositional analysis of glycosaminoglycans by SEC/MS has recently been described by Zaia and Costello [355]. The distribution of 4-O-methylglucuronic acid residues along the polysaccharide chains of xylans, isolated from various trees, has been studied by analysing the oligosaccharide mixtures obtained by partial acid hydrolysis [356]. The hydrolysates thus obtained were analyzed by MALDI-MS or by capillary electrophoresis as well as by SEC in combination with MALDI-MS. Fructans (fructooligosaccharides and inulin) originally extracted from chicory roots have been separated by continuous annular and fixed-bed conventional gel chromatography [357]. Both columns were packed with Toyopearl HW 40 (S) and eluted with de-ionized water. The productivity of the annular system was found to be 25 times higher than the conventional system. More detailed information on SEC analysis of oligosaccharides and polysaccharides can be found in Chap. 18 of this book.

5.7.5 Other applications

SEC is a very useful tool for investigating the self-association of many substances, such as surfactants, chlorpromazine hydrochloride, Methylene Blue, and a sulfobetaine derivative (CHAPS) of cholic acid [358]. The SEC of lipids has been described in several reviews [359,360]. More recently, its use in the detection of diacylglycerols and other compounds to verify the quality and authenticity of olive oil has been reported by Dauwe et al. [361]. MS of myelin proteolipids, pre-separated by means of SEC with an organic solvent as the mobile phase, has been described [362]. Humic substances have been analyzed by APCI and ESI-MS in positive and negative modes, in combination with SEC [363]. The effects of ozone, chlorine, hydrogen peroxide, and permanganate on aquatic humic matter with different molecular-size fractions, and the formation of organic acids in drinking water have been studied by SEC [364]. Aquatic humus in lake water, artificially recharged groundwater, and purified, artificially recharged groundwater were fractionated by HP-SEC/UV before and after oxidation. Fractionation of natural organic matter in drinking water by SEC and characterization by \(^{13}\)C cross-polarization magic-angle spinning NMR spectroscopy and SEC have been achieved by Wong et al. [365]. Studies of specific interactions of organic substances, such as alcohols, mono- and dicarboxylic acids, aromatic acids, and amino acids were conducted by Specht and Frimmel [366]. Adsorption of several different organic polyelectrolytes from aqueous solution by activated carbon was investigated by Kilduff et al. [367]. The polyelectrolytes studied included humic acids, extracted from peat and soil, polymaleic acid, a synthetic polymer identified as a fulvic acid surrogate, and natural organic matter in river water.

A method has been developed [368] for analyzing pesticides in dust. For non-acidic pesticides, the extract, after centrifugation and filtration, was purified by SEC and then analyzed by GC/MS. Coupling of SEC to LC/MS for the determination of trace levels of thifensulfuron-methyl and tribenuron-methyl in cottonseed and cotton gin trash has been described [369]. An on-line SEC/GC method for the detection and quantification of
organophosphorous pesticides in crude edible oils was described by Jongenotter and Janssen [370]. Other compounds, such as sterols and wax esters were also identified by an off-line technique. SEC on a Bio-Beads SX-3 column, followed by a dual GC determination has been developed by Jover and Bayona [371] for a multi-class pesticide determination in lanolin. The effluent from the analytical column (50% diphenylmethyl- or 14% cyanopropylphenylpolysiloxane) was split into an electron capture detection (ECD) and a nitrogen phosphorous detection (NPD) system. That system was optimized for 28 pesticides commonly used to control sheep pests. The molecular-mass distributions of organic poly-electrolytes remaining in solution after equilibration with various amounts of activated carbon were determined by HP-SEC. The shape separations of suspended gold nanoparticles were investigated by Wei et al., using SEC [372]. A sample of Athabasca bitumen was fractionated by preparative SEC, and the MWD of five fractions and of the original sample were determined by SEC, using a calibration based on polystyrene standards [373]. SEC on Sephacryl S-1000 has recently been used by Loa et al. to purify turkey coronavirus (TCoV) from infected turkey embryos [374].

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