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Size Exclusion Chromatography and Related Separation Techniques

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Review Contents

Size exclusion chromatography (SEC), often referred to as gel permation chromatography (GPC), is an entropically controlled separation technique in which molecules are separated on the basis of hydrodynamic molecular volume or size. With proper column calibration or by the use of molecular-weight-sensitive detectors, such as light scattering, viscometry, or mass spectrometry, the molecular weight distribution (MWD) and the statistical molecular weight averages can be obtained readily. Thus, SEC is the premier technique for determining these properties of both synthetic polymers and biopolymers. For this review, we have expanded coverage of related polymer separation techniques, including interactive (enthalpic) modes of HPLC, temperature-rising elution fractionation, and field-flow fractionation.

Important developments in SEC have continued in the area of detection systems, mainly light scattering, viscometry, and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry in conjunction with SEC for determining MW and chemical composition of polymeric materials. An emerging technology is NMR detection, which in a few years should have a major impact as an on-line detection method for SEC and HPLC.

Applications of high-performance SEC have continued to grow, especially in the area of biopolymer separations. The use of SEC for measuring physicochemical properties, especially with respect to biopolymers, has become an important area of research. There has been some incremental improvements in SEC column packings for aqueous and nonaqueous SEC packings.

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This review covers fundamental developments and selected applications of SEC and related techniques abstracted by *Chemical Abstracts* and Medline from 1996 to 1997 inclusive and is a continuation of our previous review (*A8*). Suggestions are always welcome for improving coverage of topics.

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BOOKS, PROCEEDINGS, AND REVIEWS

During this review period, only one book has been published dedicated to preparative SEC, with a focus on Sephadex LH-20 (*A1*). Conference proceedings on SEC and related techniques have been sponsored by the ACS Division of Polymeric Material Science and Engineering (*A2*), the International GPC Symposium (*A3*), the 10th Bratislava International Conference on Macromolecules (*A4*, *A5*), Rapra (*A6*), and the International Symposium on Polymer Analysis and Characterization (*A7*).

Reviews on specific SEC topics and applications are covered in the appropriate sections of this article. The Selected Applications section lists specific reviews based on polymer type. Barth and co-workers (*A8*) presented comprehensive coverage of SEC literature from 1994 to 1995; the present review is a continuation of that format. General reviews on SEC can be found in refs *A9* and *A10*. Machate (*A11*) discussed various chromatographic approaches for the characterization of coating resins, including SEC. Historical perspectives of SEC were presented by Porath (*A12*), who dealt with packings, and by Barth (*A13*) and Benoit (*A14*), who considered calibration approaches. Henry (*A15*) reviewed recent innovations in SEC and instrument design. Belenkii et al. (*A16*) described nonstandard methods based on SEC, such as critical chromatography, membrane chromatography, TLC, and microbore SEC.

THEORY

Hoagland (*B1*) presented a thermodynamic model for polymer separations by SEC, hydrodynamic chromatography, and gel electrophoresis based on the unifying principle of local equilibrium. Each of these separation methods relies on spatial variation of solute confinement to produce partitioning according to molecular weight or size when enthalpic interactions between the matrix and the solute are minimized. Under local equilibrium, the fundamental operating parameter is confinement entropy, and with curved or irregular matrix interfaces, there may not be an effective polymer radius that can correlate the elution behavior of different species. This was illustrated for several polymer and matrix models. A soft-body theory of size exclusion chromatography was presented by Potschka (*B2*). The size of the solute was considered as a distance average over the energy of interaction, which depends on the mobile phase as well as the matrix. The theory of forces was used to explain partition in SEC and the existence of universal calibration curves. A theoretical analogy between multistage ultrafiltration and SEC was developed by Prazeres (*B3*). Multistage ultrafiltration was shown to be more efficient than SEC, as similar resolution can be obtained with fewer stages.

A new type of chromatography based on stacks of ultrafiltration membranes was proposed. Brooks and Muller (*B4*) calculated the SEC partition coefficient assuming that the column packing, the gel phase, can be treated as a polymer solution of appropriate concentration and molecular weight. The mean field theory of polymer solutions was used to predict the partition coefficient of a polymer molecule distributing between the gel and mobile phases. The reduction in entropy of the macromolecule in the gel phase was sufficient to produce the experimentally observed exponential dependence of the partition coefficient K on the molecular weight of the partitioning species. The enthalpy of interaction between the gel polymer and the distributed species provided a parameter that described the specificity or recognition in the interaction.

Busnel and co-workers (*B5*) used Monte Carlo simulation to model SEC. Polymer chains of various flexibility and thickness were constructed in pores of several geometries. The steric partition coefficient K, the hydrodynamic radius R_η and the chromatographic radius R_C, defined as the radius of the rigid sphere with the same K value, were evaluated. The ratio of the two radii, R_C/R_η, was found to depend on the flexibility and relative thickness of the molecule. Boyd et al. (*B6*) used molecular dynamics simulations of oligomers in cylindrical pores to explore the reason for the failure of universal calibration at low molecular weights. The partition coefficients for oligomeric series of polyethylene, polyisobutylene, and polystyrene were computed as a function of pore size. The chromatographic, or retention, radii were found to correlate well with the radii of gyration for a given oligomeric series. However, there were significant differences in these correlations among the three oligomer series: at the same radius of gyration, the chromatographic radii were different, with polystyrene being the largest and polyethylene the smallest. The differences were in agreement with previously reported experimental results (*B7*) and were attributed to asphericity of individual configurations enhancing the effects of substituent size. The computed chromatographic radius was found to be a significantly better criterion for predicting retention time than either the hydrodynamic radius or the radius of gyration.

Packings of porous aluminas and aluminosilicates, with well-defined unidimensional regular pore structure, were used by Kurganov and co-workers (*B8*) to evaluate theoretical models of SEC. A good correlation between measured and calculated distribution coefficients was found for the simplest pore morphology, but significant deviations appeared for materials with more complex pore structure. Danilov et al. (*B9*) studied the effect of axial compression on soft packing material. The compression caused packing consolidation and elastic deformation of the gel particles, which resulted in the decrease of both the external packing porosity and the available pore volume inside the particles. A considerable decrease in retention volumes and an increase in resolution was observed in the analysis of polypeptides. Shah et al. (*B10*) used carboxyl-terminated dendritic polymers on a porous glass stationary phase to examine the permeation of small charged colloids into cavities of like charge. The experimental results for the degree of permeation were typically 20−100% larger than theoretical calculations, and reasons for these discrepancies were discussed. Sassi et al. (*B11*) compared theoretical calculations with experimental data from partitioning of poly(ethylene glycol)

and poly(ethylene oxide) on highly swollen hydrogels. Experimental SEC curves agreed equally well with theories that characterize the gel as a collection of pores or of fibers. Soria and co-workers (*B12*) modeled the stationary phase in SEC with binary eluents. The theoretical description was compared with distribution coefficients for polystyrene obtained from a silica-based packing, with benzene and methanol as the binary eluent.

Magnetic resonance imaging was used by Mitchell et al. (*B13*) to visualize the separation of proteins by SEC. Analysis of concentration profiles inside the column was used to calculate local and average intracolumn plate height values for characterization of dispersion and flow nonuniformity. The results were comparable to conventional chromatographic measurements of column efficiency and also allowed the flow nonuniformity to be observed.

BAND BROADENING

Yau and Jeansonne (*C1*−*C3*) presented a method for axial dispersion correction to molecular weights measured using the universal calibration method and SEC viscometry. Wojciechowski and Pielichowski (*C4*) introduced a new method for correcting Gaussian axial dispersion in conventional SEC and compared it to other correction procedures on experimental and simulated chromatograms. Storey and Baugh (*C5*) described the use of commercial software to deconvolute chromatograms of block copolymers.

CALIBRATION

General. Fredriksson and co-workers (*D1*) used fractions of debranched amylopectin unit chains to calibrate a size exclusion chromatograph. They found that the elution behavior of the amylopectiin was very similar to that for maltoheptaose and pullulan standards. Koenecke and Severin (*D2*) characterized deasphalted petroleum distillation residues by vapor pressure osmometry and used them as standards to calibrate a chromatograph for characterization of petroleum fractions. Molecular weight results for hydroxyl-terminated polybutadiene resins obtained from SEC using polystyrene and polybutadiene standards and vapor pressure osmometry were compared by Takahashi and co-workers (*D3*, *D4*). The SEC results depended on the standards used to construct the calibration curve. Bergstroem et al. (*D5*) used SEC to determine the amount of comonomer in an ethylene−norbornene copolymer. The two comonomers have different refractive indexes, so the refractometer peak area, for a given injected mass, varies with composition. Cook and Sible (*D6*) presented results on the effect of changes in refractive index on calculated molecular weight distributions of siloxane resins.

Mori and co-workers (*D7*) discussed the problems in determining the molecular weight distribution in chromatographs where the concentration detector baseline is not recovered between the end of the polymer chromatogram and the solvent impurity peak.

Universal Calibration. Benoit and co-workers' original paper on universal calibration (*D8*), as well as Moore's first paper on gel permeation chromatography (*D9*), were reprinted, accompanied with commentaries on the development of SEC by Benoit (*D10*, *D11*) and Barth (*D12*).

Le Maire and co-workers (*D13*) reviewed the use of SEC and universal calibration for characterizing the size and molecular mass of proteins. Although universal calibration works for water-soluble globular proteins and flexible denatured proteins, it does not apply to many detergent micelles and detergent-solublized membrane proteins nor to elongated proteins.

Chance and co-workers (*D14*) presented results from a detailed study of the breakdown of universal calibration for low-molecular-weight oligomeric species. The elution behavior of well-characterized low-molecular-weight polystyrenes, polyisobutylenes, and *n*-alkanes was investigated. Below a molecular weight of about 1000 g/mol, the universal calibration method was invalid for these oligomers, and no single calibration curve could be constructed on the basis of hydrodynamic volume or radius of gyration. In addition, the errors in molecular weight determination caused by the variation in the refractive index increment at low molecular weights were studied. If neglected, this variation can lead to errors of 10−25% in the determination of the number-average molecular weight for polydisperse materials. Mrkvickova (*D15*) also studied the retention behavior of low-molecular-weight polymers and used the Sadron−Rempp equation relating intrinsic viscosity and molecular weight, rather than the Mark−Houwink−Sakurada equation, to construct the universal calibration curve and calculate molecular weights.

Dayal et al. (*D16*) compared universal calibration with calibration based on a polydisperse standard for calculating the molecular weight averages of polypropylene. They found that, for samples with a MWD similar to that of the standard, the broad standard calibration yielded more accurate results. Universal calibration was used by Mendichi et al. (*D17*) to characterize polymeric antitumor drug carriers and by Tacx and co-workers (*D18*) to characterize poly(vinyl alcohol) using poly(ethylene oxide) standards.

ROUND-ROBIN STUDIES

Mori and colleagues in the Japan Society for Analytical Chemistry published the results of a series of round-robin studies on the determination of molecular weight averages by SEC. In the first study (*E1*), four polystyrene (PS) and two poly(methyl methacrylate) (PMMA) standards were characterized at 26 different laboratories. Each laboratory used its own experimental procedures. The relative standard deviations (RSDs) in the determination of the molecular weight averages within each laboratory were in the range 1−3%. However, between laboratories they were 16−18% for M_n and 7−10% for M_w. In the second study (*E2*−*E4*), involving the same laboratories and the same standards, the calibration standards used in each laboratory were the same, and sample concentrations and injection volumes were prescribed. The laboratories attempted to construct the calibration curves in a similar way. The RSD for M_w was 4%. Two methods of drawing the baseline were also prescribed. The first method extrapolates the baseline at the beginning of the polymer chromatogram to the baseline after the solvent peak. With this method, the RSD in M_n was 5−7%. The second method extrapolates the baseline to the end of the chromatogram before the solvent peaks. In this case, the RSD in M_n was 7−9%. Although the first method gave more precise results, a number of results were considered invalid due to baseline disturbance caused by the solvent peaks, and so the second method was considered more appropriate.

The effects of the differences in detectors and mobile phases on the calculated molecular weight averages were also studied (*E5*). Number-average molecular weights obtained for the PS samples using a UV detector were about 20% lower than those obtained using a differential refractometer. This was considered to be due to the effect of the end-structure of the PS samples on the UV absorption. The values for M_w were not affected. The PMMA standards were analyzed in chloroform in some laboratories and in tetrahydrofuran (THF) in others. Values obtained in chloroform were 5−25% higher than the values obtained in THF.

In the third round-robin test (*E6*), three PS standards were analyzed in seven laboratories using the same columns and with the same experimental conditions and procedures as the previous test. The RSDs for M_n were between 7 and 11%, with 3% RSD, for M_w. If the same person analyzed the data from all the laboratories using the same software, the RSD in M_n was reduced to 2−6%. In the fourth round-robin test (*E7*), involving 24 laboratories and three PS standards, two sets of PS calibration standards from different manufacturers were compared. The results obtained from the two calibration curves were significantly different, with molecular weight averages obtained from the curve based on one set of standards being 3−14% higher than results from the other.

Brusseau (*E8*) published a general discussion of experiences with round-robin tests in SEC. The critical points for obtaining better interlaboratory reproducibility in SEC results are discussed and include column selection, use of high-quality calibration standards, calibration procedure, selection a common detector, and uniform baseline setting procedure. Sokolov and co-workers (*E9*) also discussed the sources of discrepancy between MWD data obtained in different laboratories and described a procedure for correct comparison of the data.

Robert and co-workers (*E10*) published results of an internal validation of an SEC method for polyamides using benzyl alcohol at 130 °C as the mobile phase. They proposed that the method be used as the basis for round-robin testing of SEC analysis of polyamides. Vander Heyden et al. (*E11*) described a ruggedness test of an SEC method for low-molecular-weight polymers using a fractional factorial design. The largest source of variation was the column manufacturer and the type of detector used.

AUTOMATION/QUALITY CONTROL

Kilz (*F1*) discussed the use of SEC for quality control, as exemplified with poly(vinylpyrrolidone) and poly(butylene terephthalate). Poche et al. (*F2*) described a laboratory robotics system for high-temperature sample preparation for subsequent high-temperature SEC analysis.

PACKINGS

Inorganic-Based Packings. Buijtenhuijs and van de Reit (*G1*) demonstrated the applicability of diol-modified silica for the SEC of polyamides, PET, PBT, PVA, polycarbonate, and polyacrylonitrile. Matthijs and Schacht (*G2*) coated (aminopropyl)-triethoxysilane-treated silica with *p*-nitrophenyl chloroformate-activated dextran with subsequent hydrolysis of the remaining active carbonates on the dextran. The SEC performance of this packing was evaluated with proteins. Zhang et al. (*G3*) prepared macroporous silica of average pore size of 4×10^2−1×10^3 nm from silica gels of an average pore size of 20−30 nm by calcination. This packing was evaluated with polyacrylamide with $M_w < 1.5 \times 10^7$.

Organic-Based Packings. Danilov et al. (*G4*) studied the influence of soft-gel axial compression on packing structure and chromatographic properties. Horak et al. (*G5*) reviewed the preparation and properties of SEC packings in general. Hagel (*G6*) surveyed packings, especially Superdex (an agarose/dextran composite), for aqueous SEC with emphasis on the separation of small molecules of biological interest. The characteristics of Superdex preparative-grade packings for proteins and peptides were discussed by Hellberg et al. (*G7*). Electrostatic and hydrophobic properties, as well as stability under acidic and basic conditions, were studied. The use of Superose 12 (agarose-based packing), Superdex 75, and Superdex Peptide packings for the fractionation of complex biological feedstocks was reported by Dale and Lyddiatt (*G8*). With these packings, mixed-mode separations were also observed. The characteristics of agarose-based superporous packings were reported by Gustavsson and Larsson (*G9*, *G10*). This packing consists of two pore structures: smaller pores for normal diffusion and superpores for increased mass transfer. A patent was awarded for the manufacture of cross-linked agarose packings that are capable of withstanding higher flow rates as compared to conventional gel particles (*G11*). Ericsson et al. (*G12*) attached poly(2-hydroxyethyl vinyl ether) onto a beaded agarose matrix (Sepharose HP) and compared its performance characteristics to those of Superdex 30.

Ritter (*G13*) described the properties of a cellulose-based packing, Spherilose. The preparation of chitin particles was reported by Itoyama and Fujii (*G14*). The preparation and properties of composite metal-containing hydrogels based on dextran were given by Spychaj and co-workers (*G15*, *G16*). A cross-linked allyl dextran packing, called Acryldex, which is similar to Sephacryl, was introduced by Guo et al. (*G17*).

The separation characteristics of different particle size mixed-bed PLgel columns (cross-linked styrene−divinylbenzene) were reported by Meehan and Bartylla (*G18*). Neves and co-workers (*G19*, *G20*) described a modified suspension polymerization for producing styrene−divinylbenzene packings. Monodisperse cross-linked styrene−divinylbenzene particles, prepared using a porous-glass membrane emulsifier, were reported by Hatate et al. (*G21*). Christensen et al. (*G22*) presented an approach for attaching hydrophilic stationary phases onto styrene−divinylbenzene particles for subsequent aqueous SEC. With these packings, 10^8 MW pullan could be chromatographed. Dextran-coated styrene−divinylbenzene packings were produced and applied to proteins (*G23*).

Packings consisting of 4-hydroxystyrene−divinylbenzene were prepared and evaluated by Lewandowski et al. (*G24*) and used for both SEC and reversed-phase HPLC. SEC using polyhydroxymethacrylate gels with DMF was compared with styrene−divinylbenzene packings (*G25*). A poly(vinyl alcohol) SEC packing was synthesized from vinyl acetate and triallyl isocyanurate (*G26*).

MOBILE PHASES

Less commonly used mobile phases for SEC that have been published during this review period include dimethylacetamide

with LiCl for polysaccharides (*H1*), formamide for polybetaine (*H2*), formamide with LiCl for cationic polymers (*H3*), DMF/triethylamine/pyridine (8:1:1) for poly(4-vinylpyridine) (*H4*), quinoline for pitch (*H5*), 1-methyl-2-pyrrolidinone for coal products (*H6*, *H7*), acetone/cyclohexane (3:7) and ethyl acetate/cyclohexane (3:7) for pesticide residues (*H8*), methylcyclohexane for polypropylene and polyethylene (*H9*), formic acid for polypropyleneamine dendrimers using a reversed-phase silica deactivated by tetraazacyclotetradecane (*H10*), cyclohexane for natural rubber (*H11*), and ethanol/NaCl/phosphate for poly(propylene glycol)-modified proteins (*H12*). A micellar mobile phase has been reported for the SEC of residual acrylamide (*H13*). Vohlidal et al. (*H14*) showed that poly(phenylacetylene) degradation, which occurs within an SEC column when using THF as the mobile phase, can be prevented by using dearated THF.

Olesik's group (*H15*, *H16*) used enhanced-fluidity mobile phases for SEC of polystyrene. In these studies, THF/liquid CO_2 was employed at room temperature and moderate pressures. Although the pressure drop across the column and analysis times were reduced, significant adsorption of solutes was noted because of decreased solvent strength of the mobile phase. Just and Much (*H17*) used mixtures of methylene chloride and CO_2 to study SEC and adsorption of polystyrenes in the supercritical, subcritical, and liquid phase states. Using supercritical fluid SEC, Kuehn et al. (*H18*) characterized technical waxes, and these results were compared to MALDI-TOF/MS analysis. Beyond the MW range of 3000, the SEC approach was the method of choice.

NON-SIZE-EXCLUSION EFFECTS

Shear Degradation/Viscosity Effects. Using on-line light scattering, Nakamura et al. (*I1*) observed no shear degradation of 4×10^6 MW polystyrene during SEC. Norton and Fernandez (*I2*, *I3*) used numerical simulation and magnetic resonance imaging to study viscous fingering of protein solutions in SEC columns. Based on these investigations, a new column design was proposed.

Aqueous SEC. Han et al. (*I4*) reviewed non-size-exclusion effects in aqueous SEC. Garcia et al. (*I5*) investigated aqueous SEC of polyanions in which the separation was influenced by electrostatic and hydrophobic interactions with silica-based packings. Dubin's group (*I6*) studied protein retention on Superose 12 over a wide pH range. The effects of electrostatic and hydrophobic interactions on the chromatographic behavior of proteins with chemically bonded silica-based packings as a function of buffer composition were reported by Corradini (*I7*). Ricker and Sandoval (*I8*) presented practical guidelines for the development of reproducible SEC methods for proteins based upon optimized sample volume, flow rate, column length, and mobile phase conditions that reduce non-size-exclusion effects. Muat and Manchester (*I9*) evaluated SEC behavior of eukaryotic protein synthesis initiation factor 2 using Superose 6, Ultrogel AcA, and Sephacryl S-300. SEC behavior of amino acids on Toyopearl HW-40S was investigated by Rao et al. (*I10*). A mobile phase was optimized for the SEC of casein hyrolyzates (*I11*).

Gan and Lin (*I12*) examined the SEC performance of sodium alginate on several different packings. Zhang et al. (*I13*) studied the SEC of a polysaccharide peptide as a function of mobile phase ionic strength. SEC was used to investigate the presence of uronic acid groups on arabinogalactan (*I14*). These experiments were accomplished by analyzing samples as a function of mobile phase ionic strength: at low ionic strength, uronic acid-containing macromolecules were partially excluded. Yamamoto et al. (*I15*) described the effects of mobile phase conditions on the elution properties of galactosykojic acid using Toyopearl HW40.

Lage et al. (*I16*) discussed associative interactions and non-size-exclusion effects of chlorinated compounds of bleached kraft pulp mill effluent. SEC of lignin- and carbohydrate-containing samples, prepared from wood and pulp samples, was accomplished using alkaline mobile phases (*I17*). The underestimation of the MW of styrene−maleic anhydride copolymers, as determined by SEC, was attributed to the existence of active polar sites on the packing and partial ring opening of the maleic anhydride, which resulted in the formation of dicarboxylic acid species (*I18*). This anomalous behavior was suppressed by the addition of acetic acid to the mobile phase. Harms et al. (*I19*) evaluated non-size-exclusion effects that can occur during SEC of technetium compounds. The influence of mobile phase composition and column temperature on the distribution coefficient of technetium compounds was measured using Sephadex G-25, Zorbax GF-250, and HEMA-SEC Bio 1000.

Nonaqueous SEC. Dias et al. (*I20*) investigated non-size-exclusion effects of acrylic polymers using DMF as the mobile phase. These effects were caused by electrostatic interactions between the polyelectrolyte and ionic species from DMF degradation. This group also studied SEC behavior of acrylonitrile polymers containing ionic groups, including charged terminal end groups (*I21*). In DMA, these ionic polymers gave multimodal peaks. With the addition of LiBr, these peaks shifted toward smaller retention volumes. As in the case of DMF, this elution property was attributed to the formation of supramolecular structures by the interaction of ionic groups in the polymer and ionic species from DMA decomposition.

By adding LiBr to the NMP mobile phase, naphthalene mesophase pitch and a mixture of C_{60} and C_{70} fullerenes were adsorbed onto an SEC column and eluted after the permeation limit of the column (*I22*). Dong et al. (*I23*) studies non-size-exclusion effects using NMP with LiCl as the mobile phase. Barman (*I24*) investigated factors affecting the elution of elemental sulfur beyond the permeation limit of a cross-linked polystyrene column.

DETECTORS

Light Scattering. Wen and co-workers (*J1*) reviewed the application of SEC with on-line light scattering (LS), absorbance, and refractive index detectors to the study of proteins and their interactions. They presented a self-consistent method for combing data from the three detectors and used this method to study the stoichiometry of protein−protein interactions (*J2*). Podzimek (*J3*) and Wyatt (*J4*) presented general discussions of the application of on-line multiangle light scattering (MALS) to SEC.

Radke et al. (*J5*, *J6*) presented a method for determining the true number-average molecular weight of copolymers using SEC light scattering. The method requires that each elution increment in the chromatograph be monodisperse with respect to molecular weight and composition. Measurements on mixtures of polystyrene and poly(methyl methacrylate) indicated that the errors were

less than 10%. Cotts (*J7*) demonstrated the wide range of polymer molecular weights that can be determined by SEC with on-line multiangle light scattering. Narrow molecular weight distribution polystyrene standards, with molecular weights from 580 to 3×10^7, were characterized, and radius of gyration measurements ranging from 4 ($M_w = 24\,000$) to 400 nm were obtained. Wyatt et al. (*J8*) used radius of gyration measurements from SEC−LS to help determine the molecular weight polydispersity of narrow MWD polystyrene standards. The measured polydispersity was found to be extremely small.

SEC-LS was used by Zigon et al. (*J9*) to study the degradation of high-molecular-weight polystyrenes due to elongational shear during SEC analysis. They found no degradation below molecular weights of 2×10^6. For higher molecular weights, degradation was detected and depended on the origins and sizes of the gel particles, the porosity of the column frits, use of a precolumn filter, flow rate, and the presence of a capillary viscometer. Tanigawa et al. (*J10*) studied the changes in molecular weight and molecular weight distribution of single-, double-, and triple-stranded nucleic acids caused by sonication. Experimental SEC−LS data were compared with computer simulations and used to determine the chain-scission mechanism. Tackx and Bosscher (*J11*) used a computer simulation of multiangle LS and SEC to study systematic deviations in molecular weight determinations caused by random noise.

Micelle formation in poly(ethylene glycol) and a copolymer of poly(ethylene glycol) with lactate and acrylate groups was studied by Vilenchik and co-workers (*J12*) using SEC-MALS. Nagy (*J13*) used SEC-MALS to obtain molecular weight, radius of gyration, and conformational information for cationic and nonionic amine-functional polymers. Andrianov and Le Golvan (*J14*) characterized a water-soluble phosphazene polyelectrolyte using aqueous SEC and an on-line multiangle LS detector. The LS detector was used to study the effect of the mobile phase ionic strength and a secondary nonexclusion separation mechanism. Lee and Chang (*J15*) discussed the application of SEC-LS to the characterization of multicomponent polymer systems, and Mrkvickova (*J16*) used SEC-LS to measure the molecular weight and compositional heterogeneity of a graft copolymer.

Jumel et al. (*J17*, *J18*) used SEC-MALS to study gastric mucus glycoproteins, and Huber and Eteshola (*J19*) used SEC-LS to measure the molecular weight of an anionic polysaccharide. Fishman and co-workers (*J20*) used SEC-MALS to measure the molecular weights and radii of gyration of potato and corn starches. Molecular weight distributions of hydroxyethyl starch measured by SEC-LS were compared with those measured by conventional SEC by Mase and co-workers (*J21*). The latter method was considered more suitable for quality control. Capron and co-workers (*J22*) used SEC-LS to study xanthan and schizophyllan; the LS detector was able to identify micogels and aggregates. Knobloch and Shaklee (*J23*) measured the MWD of low-molecular-weight heparins using SEC-MALS. Light scattering detectors were used to characterize corn starch (*J24*), poly(phenylmethylsilane) (*J25*), polyesters and polyamides (*J26*), β-glucans (*J27*, *J28*), and chitosans (*J29*).

Viscometers. Lesec and Millequant (*J30*) described the development of a dual-capillary viscometer. The second capillary is used to correct for flow rate pulsations caused by the pump. Norwood and Reed (*J31*) compared a laboratory-built on-line single-capillary viscometer with a commercial bridge design viscometer. Random measurement error was significantly worse in the single-capillary viscometer, and, overall, the precision of the bridge viscometer was twice that of the capillary viscometer. The advantages of the capillary viscometer are its compactness and lower cost. For either design, a pulse-free pump is critical to realize the potential accuracy.

Poetschke et al. (*J32*) used SEC viscometry to determine the molecular weights of hyperpolymers of human hemoglobin. They also presented an iterative calibration procedure using two fractions of the polymer to be analyzed. Szesztay et al. (*J33*) used SEC viscometry to study the kinetics of radical polymerization of poly(methyl methacrylate) at high conversion. Ji et al. (*J34*) characterized segmented polyurethanes by SEC viscometry.

Combined Light Scattering and Viscometry. Reed (*J35*) reviewed the theoretical and technical aspects of combining light scattering and viscometric detectors for the characterization of polyelectrolytes. General reviews of the use of multidetector SEC instruments were also presented by Bruna (*J36*) and by Meier (*J37*). Busnel and co-workers (*J38*) described a new miniaturized, combined multiangle light scattering and viscometric detector for SEC.

Mourey et al. (*J39*) demonstrated how SEC with combined light scattering and viscosity detectors can be used to measure the local polydispersity, i.e., the width of the MWD at a single elution slice, across a chromatogram. The method was used in the analysis of polymer blends. Jackson (*J40*) studied the accuracy of molecular weight determination using a right-angle LS detector and viscometry. In most cases, measured molecular weights within 2% of the true values were obtained up to 1×10^6. Errors rapidly increased at higher molecular weights. Yau and Hill (*J41*) applied SEC-visc-LS to the characterization of brominated polystyrene and used the data to study the capabilities of each detector and the synergism in combining them into one system.

Hutchinson and co-workers (*J42*) used SEC-visc-MALS to determine the Mark−Houwink−Sakurada coefficients for copolymers of methyl methacrylate and *n*-butyl acrylate in a pulsed-laser study of penultimate copolymerization propagation kinetics in the copolymerization. Chazeau and co-workers (*J43*) used SEC-visc-LS to study the conformations of xanthan in solution, and Myslabodski et al. (*J44*) reported the effect of acid hydrolysis on the molecular weight of κ-carrageenan. Radius of gyration and intrinsic viscosity were measured as a function of molecular weight, and the persistence length was determined. The conformation of chitosan was described by Hall et al. (*J45*) using SEC-visc-LS. Havard and Wallace (*J46*) applied high-temperature SEC-visc-LS to the characterization of metallocene-catalyzed polyolefins. SEC-visc-LS was used to study hydrodynamic draining in flexible polymers in tetrahydrofuran by Jackson et al. (*J47*). An apparent dependence of the hydrodynamic parameter on chain stiffness was observed.

Multidetector SEC has been applied to the characterization of branched polymers, and these papers are covered in the Physicochemical Studies section.

Interdetector Volume in Multidetector SEC. The determination of the interdetector volume and its apparent variation

with molecular weight continues to be an active area of investigation. Thitiratsakul et al. (*J48*−*J50*) published a study of peak shape changes and the interdetector volume in SEC with multiple detectors. They found that the measured interdetector volume between the refractometer and the viscometer increased with increasing molecular weight when the detectors were arranged in a parallel configuration. The result was attributed to increasing peak skewness observed for the viscometer chromatograms as molecular weight increased. This increase in skew was not observed in the refractometer chromatograms and was not observed when the detectors were placed in series. A new method of determining the interdetector volume was presented. Zammitt and Davis (*J51*) reported a study of broad MWD standards using SEC with light scattering and viscosity detectors which highlighted the importance of accurate and precise determination of the interdetector volume in determining the true molecular weight distribution and Mark−Houwink−Sakurada constants. They found that it was necessary to correct for molecular weight dependence of the interdetector volume. Netopilik (*J52*) presented a study of the relation between the interdetector volume in multidetector SEC and band broadening. For a log-normal molecular weight distribution, the interdetector volume can be altered to correct for errors due to band broadening.

Chemiluminescent Nitrogen Detector. A chemiluminescent nitrogen detector and a UV detector were used for the SEC of food-grade protein hydrolyzates (*J53*).

Density Detector. Trathnigg et al. (*J54*) discussed the influence of molar mass, preferential solvation of polymers, and chemical composition on detector response factors.

Evaporative Light Scattering. The advantages, problems, and applications of evaporative light scattering detection for HPLC of oligomers were presented by Trathnigg et al. (*J55*). An ELSD was used for the analysis of mixtures containing fatty acids and glycerides (*J56*) and coal-tar pitches (*J57*).

Flame Ionization Detector. An FID, together with a refractive index detector, was employed for high-temperature (70 °C) SEC of petroleum waxes, with toluene or *o*-dichlorobenzene as the mobile phase (*J58*).

Inductively Coupled Plasma/Atomic Absorption Spectroscopy. SEC/ICP-MS was used for elemental speciation of trace metals in the form of large organic molecule−metal complexes in lake water (*J59*), for studying protein-bound lead in human erythrocytes (*J60*), for the speciation of cadmium (γ-glutamylcysteinyl peptide) from plants exposed to cadmium (*J61*), and for the speciation of water-soluble boron compounds in radish roots (*J62*). ICP-AES was used for detecting the copper complex of bovine serum albumin in SEC fractions (*J63*).

Tan et al. (*J64*) developed a quartz T-tube interface for coupling SEC and HPLC to an atomic absorption spectrometer. This device was used to monitor metallothionein isoforms. Laborda et al. (*J65*) evaluated an on-line electrochemical atomic absorption spectrometer for selenium speciation. In this apparatus, a flow cell was placed in a graphite furnace autosampler.

Infrared Spectroscopy. A commercially available LC/FT-IR interface device for on-line HPLC and SEC was described by Willis and co-workers (*J66*−*J68*). This detector was used for analyzing short-chain branching in polyethylene (*J69*), for mapping out the composition of styrene/butadiene copolymers (*J70*), and for HPLC analysis of polymer additives (*J71*). Cheung et al. (*J72*, *J73*) evaluated and applied their own solvent evaporation interface device for FT-IR and evaluated it with respect to polymer blends.

Mottaleb et al. (*J74*) described a heated gas-flow modified thermospray used to couple SEC to an FT-IR spectrometer. In this manner, SEC effluent was evaporated and polystyrene deposited on the surface of a moving stainless steel belt that transferred the spots into a diffuse reflectance accessory of the FT-IR spectrometer. A patent was awarded to Kallos and Papenfuss (*J75*) for the development of an FT-IR interface in which LC effluent is nebulized on a cryogenic surface under partial vacuum.

Voigt et al. (*J76*) used a flow-through FT-IR with a cell volume of 1.2 μL in the transflection mode for investigating preferential solvent effects of copolymers of maleic anhydride and styrene or methyl maleimide and styrene in THF containing water. Aust and Lederer (*J77*) redesigned a commercially available flow-through IR cell for high-temperature SEC of polyolefins. Rose et al. (*J78*) used a low-volume flow-through cell for FT-IR analysis to measure short-chain branching of polyethylene copolymers. Wu (*J79*) described an FT-IR flow-cell for SEC using carbon disulfide as the mobile phase. This detector was used to determine the chemical heterogeneity of block copolymers of SBS. Rat and Lacroix (*J80*) applied SEC coupled to FT-IR for the characterization of hydroxy-terminated polybutadiene, carboxy-terminated polybutadiene, and nitrocellulose present in propellants.

Mass Spectrometry. Guttman (*J81*) presented an approach for relating MWD data from SEC to the output of MALDI/TOF-MS. In a subsequent study, MALDI/TOF was used to investigate the MWD and the number of α-methylstyrene repeat units in SRM 1487, a PMMA reference standard (*J82*, *J83*). Danis et al. (*J84*) analyzed SEC fractions using MALDI/TOF and used this information for column calibration. This approach was also used by Montaudo et al. (*J85*, *J86*) to analyze selected SEC fractions of polydisperse polymers of PMMA and poly(dimethylsiloxane) by MALDI/TOF; the MW values were used for column calibration.

Nielen and Malucha (*J87*) collected 40 SEC fractions from each of a wide variety of polydisperse synthetic polymers and analyzed 10 of these using MALDI/TOF in the continuous-extraction linear mode. The MS data were used to generate SEC calibration curves. Raeder et al. (*J88*) used MALDI/TOF data obtained from rigid-rod tetrahydropyrene oligomers for SEC calibration. These authors also reported that fragmentation took place, as well as the formation of radical ions instead of cationic species.

Jackson et al. (*J89*) demonstrated that the most probable peak MW value, M_p, for PMMA determined by either MALDI/TOF or SEC is a function of how the data are displayed. For narrow distributions, M_p values determined by MS will be 2 monomer units smaller than the value determined by SEC. For wide polydispersity samples, M_p values from MS will be considerably lower than those values obtained from SEC. The authors recommend that M_p values should be reported for SEC (weight fraction vs log mass) and modal molecular mass, M_m, for MS (number fraction vs linear mass) data. Lehrle and Sarson (*J90*) showed that MALDI/TOF and SEC produced different MWDs from PMMA. This discrepancy was thought to be caused by polymer degradation via laser irradiation, which leads to a skewed MWD toward the lower MW region. In addition, there is preferential desorption of lower MW species, which gives progres-

sive skewing of the MW distribution toward higher MW with successive laser pulses.

Fei and Murray (*J91*) described on-line coupling of SEC with a MALDI/TOF mass spectrometer. In this approach, SEC effluent was combined with a matrix solution and sprayed directly into a TOF mass spectrometer. Ions were formed by irradiating the aerosol particles with pulsed 355-nm radiation from a frequency-tripled Nd:YAG laser. Poly(ethylene glycol) (PEG 1000) and poly(propylene glycol) (PPG 1000) were analyzed with this method. In a study reported by Kassis et al. (*J92*), SEC effluent was spray deposited onto a rotating matrix-coated substrate, and the resulting track was analyzed by MALDI/TOF. PMMA was used as the test solute with *trans*-3-indoleacrylic acid as the matrix.

Off-line SEC−MALDI/TOF measurements were reported for oligo-L-lactide (*J93*) and poly(ethylene glycol) (PEG 300) and poly(propylene glycol) (PPG 425) (*J94*).

Nielen (*J95*) used SEC/ESI-MS for the characterization of PMMA, polyester, polystyrene, and poly(tetrahydrofuran). Reconstructed ion currents and the number of oligomers observed were found to depend strongly on both the level of cationization salt and the cone voltage setting, with less polar polymers requiring a higher salt concentration in THF mobile phase. The MW data were used for column calibration. Also, the use of a tricoaxial sheath-flow interface for the postcolumn addition of cationization salt was reported. Opitek et al. (*J96*) described a two-dimensional SEC/RPLC system to separate protein mixtures from enzymatic digestions using ESI-MS as the detector.

ESI-MS was used as an SEC detector for characterizing 4-*O*-methylglucuronic acid in plant gums (*J97*), neuropeptides (*J98*), endogenous LVV-hemorphin-7 in cerebrospinal fluid (*J99*), octylphenoxypoly(ethyleneoxy) ethanol surfactant (*J100*), low-MW polyesters used in automobile finishes (*J101*), and phenol−formaldehyde resins (*J102*). Simonsick et al. (*J103*) described the use of FT ion cyclotron resonance MS for characterizing macromonomers containing glycidyl and butyl methacrylate. Atmospheric pressure chemical ionization MS was employed as an SEC detector by Rosell-Mele and Maxwell (*J104*) for characterizing metalloporphyrin classes in sediment extracts.

Nuclear Magnetic Resonance. Albert and Bayer (*J105*) reviewed on-line NMR as a detector for chromatography including SEC. Included in this review is a discussion of a new detection cell design between 5 nL and 1 μL for capillary-based separations. On-line NMR detection for HPLC, SEC, and other chromatographic methods also was reviewed by Korhammer and Benreuther (*J106*), emphasizing not only applications but also technical improvements of the technique. Eichhorn et al. (*J107*) used both on-line FT-IR and NMR for SEC of oligomeric OH-terminated poly(ether sulfone).

Osmometry. A membrane osmometer with a short response time (15 s) and a 12.2-μL flow cell was described by Lehman et al. (*J108*). This detector is based on a concentric design with a capillary-shaped membrane and has a MW cutoff below 5 000.

Ozonization Detection. An on-line ozonization device, based on the double bond analyzer, was reported by Pozniak Timoshina and Vivero Santos (*J109*). This detector, which produces ozone from oxygen passing through a UV generator, was used as an SEC detector, together with a refractometer, to obtain information about the distribution of double bonds in polymers and oligomers. Oleksy-Frenzel and Jekel (*J110*) described the simultaneous measurement of organic carbon, organic nitrogen, and organic halogens on the basis of segmental flow analysis and on-line UV oxidation. This approach was used for SEC of wastewaters.

Radioactivity Detector. Aspin et al. (*J111*) developed and applied a radioactivity detector for SEC of labeled biopolymers.

Refractometry. The refractive index detector response as a function of MW was investigated for polystyrene, polyisobutylene, poly(dimethylsiloxane), siloxane resin, and poly(ethylene glycols) (*J112*). Included in this study were IR and viscosity detectors.

Turbidity Detector. Staal (*J113*) was awarded a patent for using turbidity measurements for detecting polymers. This method was achieved by adding a postcolumn nonsolvent to the SEC effluent and monitoring the resulting turbidity.

COMPOSITIONAL HETEROGENEITY

The determination of compositional heterogeneity of polymers as a function of MW (also called chemical drift) is a vital area of polymer characterization. In this section, major separation approaches are covered: SEC with selective or specific detectors, interactive HPLC, which includes two-dimensional or chromatographic cross-fractionation methods, and supercritical fluid extraction. Temperature-rising elution fractionation, which is mainly used for determining compositional heterogeneity of polyolefins, is treated in the next section. For compositional heterogeneity studies in which the focus is on detection systems, please consult the preceding section. Also, please see the Coupled Columns/Column Switching section, below.

General. Netopilik et al. (*K1*) examined the effect of chemical compositional heterogeneity of flexible-chain binary copolymers on SEC separation. If the refractive index increments of homopolymers, whose units constitute the copolymer, are equal or close to each other, the influence of the chemical heterogeneity on the experimental data is below the detectable limit. A significant error in determining MW can occur only if the difference in the refractive index increments exceeds physically reasonable limits.

SEC with Selective Detectors. Chiantore (*K2*) used SEC with a refractometer and UV detector to determine the composition of blends of styrene−acrylonitrile (SAN) copolymer, ethylene−propylenediene (EPDM) copolymer, and EPDM-*g*-SAN copolymer. The components were separated by precipitation−redissolution LC using an ELSD. Dawkins (*K3*) and Meehan et al. (*K4*) determined the compositional heterogeneity of polystyrene−polysiloxane block copolymers and statistical butyl methacrylate−styrene copolymers via SEC with multidetectors or with on-line transfer of components to an interactive column system. Huang and Sundberg (*K5*) used a refractometer and UV detector to characterize grafting efficiency of styrene onto *cis*-polybutadiene.

A combination of a refractometer and a UV detector was employed by Xu et al. (*K6*) for determining the compositional heterogeneity of styrene in chlorinated butyl rubber/polystyrene comb graft copolymer. SEC with a refractometer and a UV detector was also used for the analysis of butadiene−styrene copolymer and polystyrene blends with polybutadiene and poly(dimethylsiloxane) (*K7*), aromatic components in lubricating oils (*K8*), and vinyl acetate functionality in vinyl acetate−vinyl alcohol copolymers (*K9*).

Interactive HPLC. Hunkeler et al. (*K10*) reviewed the development of critical conditions of adsorption and limiting conditions of solubility chromatographic methodologies. Advances in the use of HPLC for polymer and oligomer separations were presented by Lochmuller et al. (*K11*). A survey of gradient elution chromatography with emphasis on predicting retention times using cloud points and solubility parameters was given by Staal and De Swaat (*K12*). Mori (*K13*) reviewed the use of SEC and nonexclusion LC for characterizing styrene copolymers. Copolymer cross-fractionation with HPLC was summarized by Lee and Chang (*K14*). Guttman and DiMarzio (*K15*) discussed the use of mixed-solvent systems to promote adsorption for characterizing the MWD of various blocks in di- and triblock copolymers.

Berek (*K16*) outlined approaches for determining compositional heterogeneity of polymeric materials using SEC in combination with interactive modes of LC. Berek's group (*K17*, *K18*) described the coupling of full adsorption/desorption and SEC for the characterization of complex polymers. In this approach, components of a polymer mixture are selectively adsorbed and then successively and selectively desorbed into a coupled SEC column. El'tekova et al. (*K19*) determined the effect of hexane concentration in THF on the retention parameters of polystyrene and PMMA. Bartkowiak et al. (*K20*) described the mechanism of LC under limiting conditions of solubility utilizing a binary nonsolvent eluent mixture. This mechanism involves a microgradient process of exclusion, accompanied by precipitation and redissolution, which results in the elution of the polymer on the shoulder of the injection zone.

Mori (*K21*) characterized styrene−acrylonitrile copolymers by SEC, followed by stepwise gradient elution−liquid precipitation chromatography. The latter separation was performed on a C18 column, with a hexane/chloroform mobile phase in which the chloroform content was increased stepwise at 2%/5 min. Krueger and co-workers (*K22*, *K23*) coupled liquid adsorption chromatography at critical conditions to SEC for determining compositional heterogeneity of polyesters, polyethers, and other types of polymers. MALDI-TOF/MS was used for identifying fractions and for SEC calibration.

Petro et al. (*K24*, *K25*) used molded macroporous rod columns consisting of poly(styrene-*co*-divinylbenzene) as a separation medium for precipitation−redissolution chromatography of styrene oligomers and polymers. This process involves the precipitation of polymers in the column, followed by progressive elution using a simple gradient. This group (*K26*, *K27*) also reported on the use of a normal-phase HPLC packing, poly(2,3-dihydroxypropyl methacrylate-*co*-ethylene dimethacrylate), for the analysis of brominated poly(isobutylene-*co*-4-methylstyrene) and hydrophilic poly(ethylene oxides).

Meehan et al. (*K28*, *K29*) determined the compositional heterogeneity of poly(vinyl alcohol) with reversed-phase HPLC on a polystyrene packing employing a water/THF gradient. The separation was based on the degree of hydrolysis and sequence length distribution of poly(vinyl alcohol). Teramachi (*K30*) compared reversed-phase and normal-phase HPLC for determining the chemical composition distribution of poly(methyl methacrylate)-*graft*-polystyrene samples. Binary random copolymers of styrene with butadiene, methyl methacrylate, and *tert*-butyl methacrylate, and styrene−methyl methacrylate−acrylonitrile random terpolymers were separated by adsorption LC (*K31*). Sequence length as well as composition affected the elution volume of block and graft copolymers of styrene and butadiene.

Cools et al. (*K32*) used gradient elution chromatography to determine the chemical composition distribution of styrene−butadiene copolymers with a THF/acetonitrile gradient. The separation was based mainly on differences in solubility among copolymer chains with different chemical compositions. Philipsen et al. (*K33*) characterized low-MW crystalline polyester resins by gradient elution chromatography under reversed-phase conditions. The differences in redissolution between amorphous and crystalline resins were used to separate blends of both resin types by combined eluent and temperature programming.

Lee and co-workers (*K34*−K36) developed a new method for characterizing polymer mixtures in which one component is separated by SEC and the other by an interaction mechanism simultaneously using isocratic elution, the latter of which is controlled by column temperature programming. With this approach, polystyrene and PMMA were separated on a reversed-phase column. Mencer and Gomzi (*K37*) developed a column fractionation model for polymers that takes into account the use of a simultaneous solvent and temperature gradient. With this model, preparative fractionation can be optimized to obtain a desired MWD of a fractionated sample.

El Mansouri et al. (*K38*) used reversed-phase HPLC coupled to SEC to determine styrene oligomers in polystyrene packaging. Eersels et al. (*K39*) performed gradient elution LC to study transamidation in melt-mixed aliphatic and aromatic polyamides. Copolymers of acrylamide and quaternary ammonium cationic monomers were characterized by HPLC using a cyano-bonded packing (*K40*). Korotkova et al. (*K41*) used isocratic reversed-phase HPLC for determining poly(3-hydroxybutyrate) and the copolymer 3-hydroxybutyrate−3-hydroxyvalerate in microbial biomass.

In the area of oligomeric and surfactant separations, liquid adsorption chromatography has been applied to the separation of poly(propylene glycols) (*K42*), fatty alcohol ethoxylates (*K43*), α-(1,1,3,3-tetramethylbutyl)phenyl ethylene oxide oligomers (*K44*), ethoxylated oligomers surfactants (*K45*), ethoxylated nonylphenols (*K46*), nonionic poly(ethylene oxide)-type surfactant mixtures (*K47*, *K48*), and poly(tetramethylene ether) glycol (*K49*).

Supercritical Fluid Extraction. Pratt and McHugh (*K50*) used supercritical propane, butane, and dimethyl ether to fractionate poly(ethylene-*co*-acrylic acid) copolymers isothermally using increasing pressure. They were able to fractionate these copolymers with respect to chemical composition by first using a poor-quality solvent, i.e., propane or butane, that solubilizes the nonpolar ethylene-rich oligomers, followed by dimethyl ether, a strong solvent, to solubilize the acid-rich oligomers. Clifford et al. (*K51*) fractionated polyisobutylene and poly(dimethylsiloxane) with supercritical extraction using linear density programs.

Other Fractionation Methods. Monrabal (*K52*) described a technique called CRYSTAF for fractionating semicrystalline polymers on the basis of branching for polyethylene and tacticity for polypropylene. Wolf (*K53*−*K55*) reported a new method for continuous polymer fractionation based on the continuous, countercurrent removal of the low-MW fraction from a concentrated

solution. Risch et al. (*K56*) fractionated poly(*p*-phenylene sulfide) samples using a process that selectively removes low-MW species.

TEMPERATURE-RISING ELUTION FRACTIONATION

Temperature-rising elution fractionation (TREF) is used for separating semicrystalline polymers, most notably polyolefins, in terms of compositional heterogeneity, such as short-chain branching, tacticity, or comonomer composition or sequence distribution. In a TREF separation, a polymer solution is prepared at elevated temperature, because of solubility limitations, and injected into a column packed with an inert support. The flow rate is stopped and the temperature slowly lowered to a given value. During this process, the more crystalline material deposits first, followed by less crystalline (e.g., more branched) components. When the lower temperature limit is reached, the flow rate is turned back on, and the temperature is slowly increased. At this time, the fractionated polymer "layers" or phases are redissolved and detected. The resulting TREFogram thus represents the compositional distribution of the sample.

Mingozzi and Nascetti (*L1*) described a simple off-line sampling method to collect eluted TREF fractions (0.3 mg) for subsequent IR microspectroscopy and SEC. This approach was used to characterize ethylene−1-butene copolymer samples prepared by Ziegler−Natta catalysis and from homogeneous zirconium-based catalysis. This group also reported on TREF analysis for the characterization of polypropylenes (*L2*−*L4*). Aust et al. (*L5*, *L6*) compared the use of Holtrup fractionation, SEC-LALLS, and TREF for obtaining MWD and comonomer mass content of a medium-density ethylene copolymer synthesized from ethene and 1-hexane.

Aroca Hervas (*L7*, *L8*) applied TREF to a number of polyolefins, including LDPE, HDPE, LLDPE, polypropylene, and ethylene−vinyl acetate copolymers and demonstrated that TREF can serve as an alternative to ^{13}C NMR or DSC to provide short-chain branching distributions. Karoglarian and Harrison (*L9*, *L10*) used TREF to analyze ultralow-density polyethylene. Fonseca and Harrison (*L11*) showed that HDPE gave two peaks when a sample was quenched cooled in a TREF experiment.

Elicabe et al. (*L12*, *L13*) presented a mathematical analysis of the TREF fractionation process in which the distribution of crystallizable lengths (which is related to the short-chain distribution) can be obtained from the TREFogram. This thermodynamic model was used to characterize TREF fractions from low-MW polyethylenes in which lamellar thicknesses become comparable to extended-chain lengths (*L14*). Lamellar thicknesses were calculated from TREF data in which MW values of fractions were obtained up to about 142 methylenes.

Folie et al. (*L15*) described a technique called critical isobaric TREF (CITREF), used for the fractionation of poly(ethylene-*co*-vinyl). Mizu and Nagata (*L16*) were awarded a patent for TREF columns packed with polystyrene gels which was used for the characterization of LLDPE.

Karoglanian and Harrison (*L17*) reported on the similarity of compositional distribution information generated by DSC and TREF and demonstrated that DSC thermograms can be generated from TREFograms. Keating et al. (*L18*) described a DSC thermal fractionation technique for characterizing ethylene copolymers that is somewhat analogous to TREF. In this method, crystalline ethylene sequence lengths of the polymer are sorted into groups, in which the ethylene lengths are estimated using melting points of known hydrocarbons. Muller et al. (*L19*) also used a similar DSC approach to fractionate ethylene−α-olefin copolymers and compared it to TREF. Starck (*L20*) used both stepwise crystallization DSC and TREF to characterize comonomer distributions in LDPE and compared data from both techniques. Westphal et al. (*L21*) showed that, for polyethylenes of moderate to high crystallinity, DSC provided good qualitative data, while TREF was more quantitative. However, when crystallinity is low or nearly nonexistent, rheological measurements are preferable.

Selected applications of TREF are as follows: polypropylene (*L22*−*L27*), maleic anhydride-grafted impact-resistant polypropylene (*L28*), propylene−butene copolymer (*L29*), ethylene−propylene block copolymer (*L30*, *L31*), ethylene−butene copolymer (*L32*, *L33*), HDPE (*L34*), LLDPE (*L35*), and ethylene−styrene copolymer (*L36*). There also have been a number of patents issued that include specific TREF data to help establish polyolefin composition (*L37*−*L58*). These patents may be of interest to those actively working with TREF.

PHYSICOCHEMICAL STUDIES

Synthetic Polymers. (a) Branching. Bahary and Hogan (*M1*) evaluated methods for determining the degree of long-chain branching in polysaccharides by SEC-visc-LS. The amount of branching was determined from both the radius of gyration measured by MALS and the intrinsic viscosity. The results agreed well with each other and with results from methylation and NMR studies. Jackson et al. (*M2*) used SEC-visc-MALS to study random branching in copolymers of methyl methacrylate and divinyl benzene. The radius of gyration and intrinsic viscosity branching parameters, g and g', respectively, were measured and compared with theoretical predictions.

Kohjiya and co-workers (*M3*−*M5*) used SEC to study the size distribution of polymer clusters in the pregel state during network formation. The experimental chromatograms were compared to theoretical ones based on the Flory−Stockmayer theory and Gaussian chain distributions. In general, at high extents of conversion, a long, high-molecular-weight tail was found in the experimental chromatograms that was not predicted by the theory. This suggests the formation of high-molecular-weight linear structures due to inequal reactivies of the cross-linker functional groups caused by steric hindrance by the reacted sites. Pille et al. (*M6*) used SEC with DRI, UV, and MALS detectors to study microgel formation in the reaction of living poly(butylstyrene) and dimethacrylates. MALS was used to measure molecular weight and determine branching, and the RI and UV detectors were used to measure composition as a function of molecular weight.

Lesec and Millequant (*M7*) analyzed star-branched copolymers using SEC-visc-LS. The branching ratio g' was measured, and the number of arms on the stars was calculated from the ratio of the number-average molecular weight of the star to the number-average molecular weight of the arm. The values of g' were compared with theoretical predictions. Liu et al. (*M8*) presented a novel method for characterizing the number of arms on a star-shaped styrene−butadiene block copolymer using only a concentration detector. The results were compared to those obtained

by LS and were in good agreement. Frater et al. (*M9a*) used SEC-visc-MALS to determine the kinetics of formation of a chlorosilane-linked polystyrene six-arm star. They also studied divnylbenzene-linked polystyrene stars with mixed arm lengths and presented evidence for coupled stars (*M9b*). Spinu and co-workers (*M10*) used SEC-visc-MALS to characterize stars formed from stereoblock copolymers with alternating amorphous and semicrystalline poly(lactic acid) blocks.

Striegel and Timpa (*M11*) showed how universal calibration and on-line LS could be applied to characterize a number of different polysaccharides in terms of molecular weight distribution and branching. Yoshikawa and co-workers (*M12*) used SEC-MALS to measure the molecular weight distribution and branched structure of biodegradable aliphatic polyesters, and Wakabayashi (*M13*) used SEC-LS to characterize branching in linear low-density polyethylene.

(b) Adsorption Studies. Kilduff et al. (*M14*) used SEC to study the adsorption of humic acid, poly(maleic acid), and natural organic matter from river water on activated carbon. The MWD of polyelectrolytes remaining in solution after equilibrium with activated carbon were measured. Kaczmarski et al. (*M15*) evaluated competitive adsorption of a nonioinic surfactant and nonionic hydrophobe-modified ethoxylated urethane thickener on pretreated rutile titanium dioxide pigments with SEC.

(c) Association Studies. Munk (*M16*) reviewed classical methods, including SEC, for studying micellization and thermodynamics of micellar phenomena of block copolymers. SEC was used to study micellar solution solubilization of benzyl alcohol in epoxy ethane−epoxy propane−epoxy ethane tripolymer micellar solution (*M17*, *M18*). This method was also used to monitor micelle−molecular equilibrium. Patrickios et al. (*M19*) employed aqueous SEC to probe the aggregation behavior of random, diblock, and ABC triblock methacrylic polyampholytes. Piccolo et al. (*M20*) studied the micelle-like conformation of humic substances using SEC.

(d) Electric Double-Layer Studies. Fischer and Kenndler (*M21*) used SEC experiments to derive the electric double-layer thickness and its ionic strength dependence of CdS colloids. This work was accomplished by measuring retention volumes of colloids as a function of mobile phase ionic strength.

(e) Kinetic and Degradation Studies. Buback and Laemmel (*M22*) described a novel type of pulsed laser polymerization SEC experiment for determining free radical propagation and transfer rates for butyl methacrylate. Hungenberg et al. (*M23*) used the pulsed-laser polymerization technique to determine propagation rate coefficients from MWDs resulting from intermittent initiation.

Using SEC, Goto and colleagues (*M24*−*M26*) studied the mechanism and kinetics of activation processes in a nitroxyl-mediated polymerization of styrene. Yan et al. (*M27*) used SEC to follow anionic oligomerization of styrene initiated by butyllithium. Krol (*M28*) developed kinetic models of progressive polyaddition for the synthesis of polyurethanes and verified the model experimentally with SEC. The grafting reaction of oligodeoxyribonucleotides on *N*-vinylpyrrolidone/*N*-(acryloxy)succinimide copolymers was investigated by Erout et al. (*M29*) using SEC and free solution capillary electrophoresis. Kidera et al. (*M30*) described an SEC procedure for studying polymer network formation as a function of conversion for the cross-linking reaction between a bifunctional prepolymer and a trifunctional cross-linker.

The hydrolytic rate constants of poly(ortho ester)s undergoing random scission were determined using SEC and NMR (*M31*). Sedlacek et al. (*M32*) employed SEC to study autoxidation degradation of substituted acetylene polymers. This group also discussed random degradation of poly(phenylacetylene) in SEC columns (*M33*). Clay and Koelling (*M34*) evaluated extensional degradation of concentrated polymer solutions by the use of rheological properties and SEC. The degradation kinetics of poly-(2-hexyne) membranes was modeled via SEC by Gonzalez-Velasco et al. (*M35*).

Aoyama (*M36*, *M37*) monitored decomposition of plant residues in soil with SEC. Singh and Ali (*M38*) studied the degradation of different acid-modified starches by SEC.

Biopolymers. (a) Structure/Conformation Studies. Estimation of the molecular mass and/or hydrodynamic size of peptides, proteins, glycoproteins, carbohydrates, and complexes by SEC has become commonplace in the biochemistry laboratory. Rapid and convenient determinations can be conducted using high-resolution HPLC (FPLC) columns. A number of examples of the estimation of protein molecular mass are included in the section on Selected Applications. The ready availability of recombinant proteins has shifted the former USE OF SEC predominant for high-abundance proteins to its much broader use for a variety of proteins now available as recombinant bacterial expression products. This change in approach offers the opportunity to study proteins which were not available previously (due to their low abundance in an organism) and to examine the effects of specific amino acid substitutions (site-directed mutants) on protein structure, function, and stability.

Analysis of the oligomeric structure of native proteins, enzymes, and complexes can be accomplished by SEC determination of molecular size under native conditions, followed by comparison of molecular mass of subunits using dissociating (denaturing) conditions or knowledge of polypeptide mass based on sequence information. The determination of dissociated (denatured) subunit molecular mass can be achieved by SEC, for example with a mobile phase containing a chaotropic salt like 6−8 M guanidine-HCl (GuHCl) or urea, or an ionic detergent, such as 0.05−0.2% sodium dodecyl sulfate (SDS), by using SDS polyacrylamide gel electrophoresis (SDS−PAGE), or, increasingly, by mass spectral analysis of dissociated subunits.

Subunit analysis of proteins can be relatively straightforward for aqueous buffer-soluble proteins and enzymes, and many examples are available in the literature. Selected examples include the determination of the homotetrameric subunit structure of bovine testicular PA phospholipase A (*M39*), the S-100B β-subunit homodimers (*M40*), and site-directed mutants of the β-subunit (*M41*), the p66/p51 HIV-1 heterodimer, and dimer-destabilizing mutants of the p66 subunit (*M42*). The complex formed between tissue-type plasminogen activator (tPA) B chain and plasminogen activator inhibitor type 1 (PAI-1) was characterized by SEC (*M43*) in two steps: (1) isolation of the complex under native conditions, to establish the identity and stiochiometry of the complex, and to remove any impurities, and (2) separation in acidic acetonitrile to dissociate the complex. These later conditions revealed the covalent interaction of tPA and PAI-1 and, additionally, showed a PAI-1 C-terminal peptide fragment of 33 amino acids, which was

liberated and resolved from the complex using the denaturing SEC conditions.

SEC was employed to define the effects of replacing specific histidine residues in recombinant sheep liver cytosolic serine hydroxmethyltransferase on dimer subunit interactions, in relation to the cofactor-binding and catalytic reaction sites (*M44*). Tryptophan hydroxylase (TPH) of rabbit is a homotetramer of 444-residue subunits. Mutant TPHs with deletions of 8, 12, and 17 residues of the C-terminus yielded fully active enyzme, although all of the mutant TPHs shifted to monomers, determining this C-terminal region to be a subunit binding domain and proving that the catalytic activity of the enzyme is not dependent on the formation of the tetrameric structure (*M45*). Shrimpton et al. (*M46*) used SEC and site-directed mutagenesis to investigate the disulfide bond-mediated reversible oligomerization and catalytic activity of the metalloendopeptidase EP24.15. The high activity of monomeric enzyme, relative to that of dimers, trimers, and higher order oligomers, demonstrates a novel mechanism for regulating enzyme activity. These results contrast with the increasing nuclease activity of RNAase A oligomers toward double-stranded RNA (*M47*). Attempts at SEC analysis of the subunit stoichiometry of Tac-soluble recombinant interleukin 2 receptor displayed ionic exclusion elution characteristics under a variety of mobile phase and stationary phase conditions (*M48*). A combined SEC immunochemical approach to determining the number of monoclonal antibody-binding epitopes per protein, called stoichiometry-ordered size (SOS) analysis, suggested the correct structure of the receptor as a monomer. This result was elegantly confirmed by both sedimentation equilibrium and sedimentation velocity studies and by circular dichroic spectral analysis. The nonideal SEC elution behavior was rationalized by considering the probable effects of the large amount of protein-linked carbohydrate on this protein (>25% by mass).

SEC analysis of the subunit structure of membrane proteins remains a challenging undertaking. To determine the oligomerization state of bovine liver monoamine oxidase B (MAO B), Shiloff et al. (*M49*) conducted SEC of purified enzyme in octyl glucoside and Triton X-100R solutions. Comparison of enzyme-specific activities and elution profiles showed that the minimal stable structural unit is a dimer, and the minimal active enzyme is the tetramer. Higher order oligomers are formed from MAO B dimers, exhibiting cooperativity of increasing activity with increasing mass, up to the size of the hexamer or octamer. The hexamer appears to be the physiologically relevant form of the enzyme. To determine the structure of the membrane domain of red cell membrane band 3 protein (MDB3), SEC was conducted in mixtures of phospatidylcholine and the $C_{12}E_9$ detergent (*M50*), revealing the native structure as a detergent−lipid complex with the protein dimer. The MDB3 dimer was dissociated to the monomer only under denaturing conditions.

Since the determination of the oligomerization state of the leucine zipper-like domain of the HIV-1 transmembrane protein gp41 using synthetic peptides was unsuccessful, Shugars et al. (*M51*) used a recombinant chimeric protein combining this region fused to the highly soluble maltose-binding protein (MBP). SEC analyses, analytical ultracentrifugation, light scattering, and chemical cross-linking methods demonstrated that the normally monomeric MBP formed tetramers when fused to the coiled-coil-forming leucine zipper-like domain, but not when a critical isoleucine of the domain was replaced by an alanine residue. SEC analysis using 1% Triton in the mobile phase demonstrated that plasma membrane-associated protein Eps15 (*M52*) readily forms high-order aggregates either as the cellular protein or when expressed as a recombinant protein. The combination of SEC and SDS−PAGE of chemically cross-linked Eps15, and deletion mutants thereof, determined that the protein exists as dimers and tetramers (and possibly higher order dimeric multiples), which are strongly associated through the central coiled-coil region of the protein.

SEC is an effective tool for the determination of the hydrodynamic properties of a particle, being sensitive to shape as well as molecular size. Wiech and colleagues (*M53*) used SEC under native conditions to define the asymmetrical nature of the algal, yeast, and human centrin proteins. The properties of these calcium-binding proteins were compared to those of the distantly related yeast calmodulin, also an EF-hand calcium-binding protein. Unlike calmodulin, these calcium-binding proteins show an extended conformation, which becomes more compact in the presence of calcium. Also unlike calmodulin, the centrins display a tendency to form oligomers in the calcium-bound state. The kinase inhibitor protein $p21^{Waf1/Cip1/Sdi1}$, and the A and F fragments thereof, also display an extended conformation by SEC, relative to globular protein standards (*M54*), and can be readily fragmented by proteolytic enzymes thoughout its structure. Both observations support the notion that this protein exhibits little secondary or tertiary structure under the conditions of analysis. SEC analysis of the 108 amino acid residue α1 single-motif spectrin peptide determined a folding unit with an elongated conformation, consistent with a rodlike shape (*M55*).

The molecular size and conformation of mucus glycoprotein (mucin) was investigated by the combination of SEC with MALLS, analytical ultracentrifugation, and intrinsic viscosity determination (*M56*, *M57*). Intact "native" mucin exhibited a weight-average molecular weight of 5.5×10^6, which dropped to 2.1×10^6 upon reduction (subunits) and 0.6×10^6 on digestion with papain (basic units). Evaluation of the Mark−Houwink equation revealed the exponent of 1.1, suggesting a linear random coil model for colon mucin, which is composed of about three subunits, which are in turn composed of three or four basic units. Analogous experiments were conducted on polymerized hemoglobins and myoglobins (*M58*, *M59*), formed by chemically cross-linking the proteins. Evaluation of the Mark−Houwink relationship revealed exponents of 0.39 for haemoglobin polymers and 0.46 for myoglobin polymers, suggesting intermediately structured polymers with a "branchlike" character.

The properties of native and renatured xanthans were studied by SEC combined with online MALLS and viscosity detectors (*M60*) and the measurements combined with offline MALLS measurements. Native xanthans were defined as those obtained by dissolution at moderate ionic strength; renatured xanthans were briefly heated at 80 °C and then cooled. No significant differences in MWD were induced by the renaturation treatment, suggesting that the renatured xanthans were either not fragmented or randomly aggregated. The renatured molecules were much stiffer, with persistence lengths of about 1000 Å, versus 300 Å, and the mass per unit length shifting from 98 to 200 Da/Å in the

renatured sample. The authors interpret these findings to support the model of a double-standed helical form for the renatured xanthan, compared to a single helical form for the native material.

(b) Association and Protein Folding. As was apparent in the previous review period, SEC analyses have contributed considerably to characterizing protein and peptide folding, association, and aggregation kinetics. These topics are intimately related, especially in cases where the studies address folding patterns of multisubunit proteins, where monomeric proteins exhibit a folding intermediate which passes through a self-associable state, or when aggregation may involve a partly unfolded polypeptide. The driving force for studies in this area includes the abundant availability of proteins expressed by recombinant DNA techniques and of smaller protein fragments generated by improved peptide synthesis, as well as growing knowledge of the patterns of protein folding and subunit associations. SEC is of considerable use in those cases where the object of the study is to determine the kinetics or thermodynamics of oligomer or conformer formation, or when "stable" protein-folding intermediates can be characterized and/or isolated.

The oligomerization state of PDC-109, the major heparin-binding protein of bull seminal plasma, was observed by SEC to be strongly affected by the availability of phosphorylcholine and to vary with the concentration of mono- and divalent cations (*M61*). SEC analysis of the 40 amino acid human β-amyloid peptide [Aβ(1−40)], a radio-labeled derivative, and two fluorescent-tagged derivatives determined the peptide to exist as a dimer at low concentrations (*M62*); the dimeric structure of the synthetic peptide was confirmed independently by others (*M63*) and is in agreement with the structure observed by Roher et al. (*M64*) for the peptide extracted from human tissue. The kinetics of oligomerization (fibrillogenesis) of Aβ(1−40), Aβ(1−42), and variants of Aβ(1−40) were studied by SEC on a variety of stationary phases, using several mobile phases, and correlated with measurements by quasielastic light scattering spectroscopy (*M65*). Chan et al. (*M66*) studied the association of Aβ(1−40) and Aβ(1−42) with recombinant apolipoprotein E variants, detecting a rapidly formed Aβ_4ApoE$_4$ complex and a slowly forming HMW complex.

Oligomerization of proteins requires specific regions of contact between subunits. The analysis of the aggregation state of multisubunit proteins by SEC, and of mutant polypeptides which have deleted or substituted sequences, can yield information on sites of subunit contact, provided that such experiments are not complicated by the possibility of global conformational changes in the folded subunit. Thus, studies of protein associations are rarely conducted using a single analytical method to determine the quarternary structure of proteins in solution but are usually combined with spectroscopic and analytical ultracentrifugation experiments.

The effect of amino acid substitutions in 14-residue synthetic peptides, designed to form amphipathic α-helices, on the formation of dimers and monomers was investigated by Houston et al. (*M67*). The positioning of alanine residues in peptides designed to form two- or four-stranded parallel or antiparallel coiled-coils strongly affected the formation of the appropriate oligomers (*M68*). Synthetic peptides corresponding to various segments of the human kinesin neck region were examined by SEC-MALLS to determine the sequences required for the formation of stable α-helical coiled-coils (*M69*). A similar approach was employed to examine the amino acid requirements at surface positions which encouraged dimer associations of helical peptides, designed on the basis of the GCN4-p1 peptide (*M70*). The type I collagen peptide fragments generated by cyanogen bromide treatment (*M71*) formed dimers and trimers in equilibrium with monomers; the associations and conformation of the oligomers varied with temperature and ionic strength. A small, globular, water-soluble protein which forms pores in lipid bilayers (*M72*) was designed, synthesized, and characterized by SEC.

Catalytically active human endothelial nitric oxide synthase (eNOS) is a dimer which is activated by association of calcium-bound calmodulin. To determine the features of the activation of eNOS by calmodulin, a truncated recombinant N-terminal oxidase domain of eNOS was developed. Using SEC, this eNOS oxidase domain was observed to be monomeric in the presence of calcium-free calmodulin but dimerized in the presence of both calcium and calmodulin, identifying the oxidase domain as the site of dimerization and of calmodulin association (*M73*).

The sites of interaction of the human invariant chain polypeptide (I chain) with the human major histocompatibility complex class II $\alpha\beta$ heterodimer were investigated by preparing proteolytic and recombinantly derived fragments of the I chain (*M74*) and then determining the ability of the derived I chain fragments to form the trimeric heterotrimer complexes (analogous to the wild-type $\alpha\beta$I nonameric complex). Identification of the interacting complexes was accomplished by the combination of SEC isolation, chemical cross-linking, and immunochemical identification of both in vivo and in vitro synthesized complexes.

Exocytosis of synaptic vesicles involves a complex collection of proteins, many of which are membrane-associated. The SNARE proteins are intimately involved in this exocytotic process, and the properties and associations of three of these proteins, SNAP-25, syntaxin, and synaptobrevin, have been investigated by Fasshauer et al. (*M75*, *M76*), using recombinant soluble domains of the latter two proteins, truncated to remove the transmembrane regions. SEC with MALLS detection of the syntaxin/SNAP-25 binary complex reveals a 2:1 stoichiometry, which shifts to a 1:1:1 ternary complex on addition of synaptobrevin. The authors note that MALLS detection is required in order to obtain correct molecular mass of the individual proteins, as well of the complexes, due to the extended conformation of these proteins relative to the globular protein standards used to calibrate the SEC system.

The chaperonins are a series of proteins which assist in the normal in vivo processes of nascent polypeptide translocation, folding, and assembly. SEC has been heavily used to study chaperonin polypeptide structure and conformation, associations with other chaperonins, and the associations between chaperonins and unfolded or partially folded substrate polypeptides. Mammalian Hsc70 is a constitutively expressed molecular chaperone protein which self-associates, associates with cochaperones of the DnaJ family, and binds to unfolded polypeptides. The oligomerization of Hsc70 and its N- and C-terminal domains was studied by SEC and analytical ultracentrifugation (*M77*). The intact protein and the C-terminal domains exist in a concentration- and temperature-dependent equilibrium mixture of monomers, dimers,

and trimers, whereas the N-terminal domain remains monomeric under all conditions examined.

Jiang et al. (*M78*) studied the association of Hsc70 with the DnaJ homologue, auxilin, and the J-domain thereof. Both intact auxilin and the 70 residue J-domain polypeptide bound to Hsc70 stoichiometrically to support the oligomerization of Hsc70. SEC conditions were developed to slow the auxilin-Hsc70 complex dissociation sufficiently to permit determination of the dissociation constant by Scatchard analysis. The association of chaperone SecB from *Escherichia coli* with denatured protein ligands was investigated by Topping and Randall (*M79*). Complex formation occurs only between SecB and unfolded polypeptides, and the binding of unfolded ligands is readily reversible. The equilibrium between bound and free unfolded ligands was shown by SEC analysis of mixtures, which shifted the refolding kinetics to favor the formation of refolded (and, therefore, nonbinding) ligand.

The interactions of *E. coli* GroEL with native cytochrome *c*, porphyrin cytochrome *c*, *apo*-cytochrome *c*, and three fragments of the protein were studied by SEC using various mobile phase salt concentrations, revealing the importance of substrate solution structure on the ability of this chaperonin to bind the ligand (*M80*). The α-crystallin lens protein functions as a molecular chaperone. The hydrodynamic properties and structure of oligomeric bovine lens α-crystallin were investigated by the combination of SEC, light scattering, and analytical ultracentrifugation in the presence of SDS and dodecyltrimethylammonium bromide (*M81*). Das et al. (*M82*) studied the conformation, aggregation state, and chaperonin activities of α-crystallin in response to elevated temperature. The interaction of α-crystallin with variously manipulated conformational forms of bovine α-lactalbumin was studied by SEC and proton NMR (*M83*). The oligomerization state of *Caenorhabditis elegans* HSP16-2, a small heat shock protein structurally related to α-crystallin, was examined by SEC, as were several recombinant variants of HSP16-2 (*M84*).

Reversible associations between proteins is a critical means for controlling their biological function. The associations can be between identical subunits (homooligomers) or nonidentical subunits (heterooligomers) or can be intact proteins interacting with other proteins or biological macromolecules, thereby forming supramolecular complexes. SEC analysis of such complexes and their components can be of great benefit.

The great success in developing therapeutic agents targeting the dimeric HIV protease has generated considerable interest in the properties of other viral proteases. Analysis of the quarternary structure of the human cytomegalovirus protease was conducted by SEC, analytical ultracentrifugation, and steady-state enzyme kinetics, demonstrating that the protein exists in a monomer−dimer equilibrium, in which the dimeric protein is the active enzyme (*M85*). The dissociation of the active protease is sensitive to solution conditions, with increasing glycerol favoring the dimeric form of the enzyme (and thus maintaining higher specific activity). Essentially the same properties were subsequently found to be true for the herpes simplex virus type 1 (HSV-1) protease (*M86*). The active HSV-1 protease is a dimer in equilibrium with an inactive monomer, and the dimeric protein is favored in solutions containing glycerol and other antichaotropic solvent additives (citrate and phosphate buffers).

The binding of many polypeptide growth factors, hormones, and cytokines occurs with, or can bring about, dimerization or higher order oligomerization of receptor proteins and coupled effectors. The best known example of this behavior is human growth hormone binding and subsequent membrane receptor dimerization. SEC analyses of receptor complexes, or soluble mutant derivatives of membrane receptors, give insight on the stoichiometry of ligand binding and define protein structures which may impact ligand binding and receptor associations. Many examples of the use of SEC to analyze receptor interactions and receptor multimerization have been reported during the review period, including binding of ovine placental lactogen to rat lymphoma cell prolactin receptor and the formation of the receptor dimer (*M87*); recombinant human stem cell factor (and mutants) binding to soluble extracellular domain fragments of the Kit receptor and its derivatives (*M88*, *M89*); estrogen receptor ligand binding to a soluble C-terminal binding domain of the receptor, dimerization of the binding domain, and its dissociation kinetics (*M90*); association between brain-derived neurotrophic factor and a truncated immunoglobin domain of the human neurotrophin TRKB receptor (*M91*); and soluble recombinant HEK receptor interactions with LERK3- and LERK7-FLAG fusion ligands (*M92*). SEC-MALLS was applied by Odaka et al. (*M93*) to the study of epidermal growth factor (EGF) to a soluble recombinant extracellular receptor domain (sEGFR). The receptor is a monomer which forms the dimer on binding to EGF, forming the EGF_2-$sEGFR_2$ complex. Large-zone SEC experiments permitted the determination of the dissociation constant for the EGF/sEGFR complex. The binding of interleukin-6 (IL-6) to the soluble extracellular domain of its receptor (sIL-6R) forms a binary complex, which associates with the gp130 protein, or its soluble variant, spg130, to form the ternary complex, IL-6/sIL-6R/sgp130, which in turn is dimeric. Hammacher et al. (*M94*) compared the ability of IL-6 and a mutant, (QT)IL-6, to bind to sIL-6R, forming the binany receptor complex, and to form the hexameric ternary complex.

Wyatt et al. (*M95*) studied the association and dissociation kinetics of tetramer formation for the G-quartet oligodeoxynucleotide d(TTGGGGTT). Using SEC to distinguish the monomer and tetramer, the kinetics were analyzed at varying temperatures from 5 to 65 °C in phosphate-buffered saline. Comparison of the phosphodiester and phosphorothioate oligonucleotides showed an association rate that was about 10-fold faster for the phosphodiester and a dissociation rate that was about 10-fold slower. A model of the association reaction features a rapid association/dissociation of monomer/dimer with a rate-limiting dimer/tetramer association.

Current thoughts on protein folding pathways recognize that a variety of schemes may apply to describe the transition of unfolded polypeptide (U) to the native folded structure (N). Evidence continues to accumulate on the existence of folding intermediates for many proteins. The existence of the intermediate states will generally be shown under specific environmental conditions to stabilize the intermediate. The best known of these is the molten globule (MG) state, which is also variously known as the "compact intermediate", or "collapsed form". Other intermediate states (I) are known to exist. A number of model schemes have been described in recent studies using SEC for

the analysis of protein folding, including

$$N \rightleftarrows U \quad (1)$$

$$N \rightleftarrows MG \rightleftarrows U \quad (2)$$

$$N \rightleftarrows MG \rightleftarrows I_x \rightleftarrows U \quad (3)$$

where I_x respresents intermediate state(s) and $x = 0...n$. Associations or aggregates may also form, complicating these reactions:

$$\begin{array}{ccccc} N & \rightleftarrows & MG & \rightleftarrows & I_x \rightleftarrows U \\ & & \downarrow\uparrow & & \downarrow\uparrow \\ & & (MG)_a & & (I_x)_a \end{array} \quad (4)$$

where subscript a represents associated species. Similarly, multisubunit proteins may exhibit much more complex folding equilibria, e.g., with a homodimer, the simplest scheme would be

$$N_2 \rightleftarrows 2I_x \rightleftarrows 2MG \rightleftarrows 2I_y \rightleftarrows 2U \quad (5)$$

where subscript $x, y = 0...n$, and the intermediates I_x and I_y are different. Experimentally, protein folding patterns are frequently investigated by physiochemical analysis of equilibrium intermediates formed by varying the concentration of denaturing agents, such as SDS, urea, or guanidine-HCl (GuHCl), by altering the pH (particularly to low pH), or by using high-temperature treatments.

Many previous studies have proven the utility of SEC for analyzing the two-state transition of monomeric proteins, as represented by eq 1. In general, it appears unlikely that this model represents the case for many proteins. Even in the simple cases of smaller, single-domain proteins, there usually exists an intermediate unfolding state. The study of the GuHCl-induced unfolding of adenylate kinase by Zhang et al. (*M96*) illustrates the need to closely examine the changes in SEC unfolding profiles and to combine SEC data with other measures of conformation, to evaluate the existence of folding intermediates. In this study, the presence of two folding intermediates was surmised, although either the interconversions of the species during chromatography or their similarity of molecular size does not permit their distinction by SEC. Similarly, the acid-unfolded form of equine β-lactoglobulin was found have a hydrodynamic radius as compact as that of the native form, although clearly it exists as a distinct conformer, as proven by near-UV CD and NMR spectroscopies (*M97*). The structured compact nature of the intermediate and its tendency to aggregate are consistent with the definition of the MG state (as in eq 4, above). Human plasma vitronectin folding and refolding also exhibit an MG-like intermediate state which readily forms HMW multimers (*M98*). The equilibrium between the native fully folded monomers and oligomers is strongly affected by the refolding conditions of ionic strength and redox potential. The unfolding characteristics of recombinant human interstitial collagenase in GuHCl are such that two chromatographically distinct intermediates could be observed and characterized by SEC and spectrocopic measures (*M99*). Other studies proving the existence of folding intermediates and their hydrodynamic and aggregative properties included two rather stable intermediates present in firefly luciferase refolding in GuHCl solutions (*M100*), the dimeric intermediates of colicin E1 channel peptide (*M101*), human cystatin C (*M102*), and *E. coli* Trp repressor fragments (*M103*), and the helical tetrameric bundles of ovine corticotrophin-relasing factor, formed by heat treatment (*M104*).

The production of biologically active recombinant human stem cell factor (rhSCF) from reduced and GuHCl-denatured polypeptide was investigated in detail by Jones et al. (*M105*). Native rhSCF is a dimer, requiring two intramolecular disulfides; thus, formation of the native structure requires both sulfhydryl oxidation and refolding reactions. At least five intermediate folding forms are involved in the refolding reaction; these intermediates can be isolated by reversed-phase HPLC and their hydrodynamic properties characterized by SEC with LALLS. Two of these intermediates, I_4 and I_5, are disulfide-linked dimers, having comparatively little structure and little significance in the folding pathway. Intermediate forms I_1 and I_2 are correctly disulfide bridged, while I_3 is not. SEC with LALLS detection determined that all I_1, I_2, I_3, and reduced rhSCF all exist as dimers, suggesting that dimerization precedes disulfide bond formation during folding. Analysis of the monomer/dimer equilibrium of rhSCF and several variants derived association constants in the range of $(2-4) \times 10^8$ M^{-1}, suggesting that the physiologically relevant form of SCF may be the monomer (*M106*). Determination of the oligomerization state of rhCSF in spiked human plasma showed a significant fraction of monomer at 10−100 ng/mL total SCF.

SEC-MALLS was applied to a detailed study of the kinetics and thermodynamics of the association of variant *E. coli* aspartate receptor cytoplasmic fragments (*M107*). The two mutant fragments were distinguished by subunit stoichiometry, forming dimeric or trimeric proteins. Both mutant proteins were in equilibrium with their monomers, reversibly dissociating to monomers which exhibited reduced tertiary structure. Analysis of the temperature dependence of the dissociation kinetics suggested a largely unfolded transition-state intermediate.

The study of the unfolding and refolding of wild-type and mutated human nucleoside diphosphate (NDP) kinase A was undertaken to define the relationship between the proteins structure and the serine 120 to glycine point mutation (S120G) associated with aggressive neuroblastomas (*M108*). SEC analysis of renaturation from urea solutions of the wild-type kinase yields native hexameric enzyme, whereas refolding of S120G yields little or no hexameric enzyme, exhibiting an accumulation of a folding intermediate with a hydrodynamic size between those of the folded monomer and the unfolded monomer. The size of the intermediate, as well as its enhanced binding of ANS (a fluorescent reporter), suggests it to be a molten globule intermediate. In an analogous manner, Eftink and Ramsay (*M109*) studied the unfolding of staphylococcal nuclease A and two low-stability mutants, NCA and NCA S28G. SEC analysis of unfolding confirmed the lower stability of the mutants in the presence of GuHCl and urea or to elevated temperatures. Analysis of the NCA S28G protein at low temperatures, between 5 and −1 °C, determines a unique "cold unfolded" folding intermediate, which exhibited a hydrodynamic volume larger than the native structure but smaller than the fully unfolded enzyme. This mutant also exhibited a high-temperature partially unfolded form, which could be fully unfolded upon the addition of urea. Both of these studies of "simple" mutations illustrate the complex rules governing the

relationships between protein primary structure and protein folding.

The presence of ligand frequently will stabilize proteins, i.e., reduce their denaturation, under a variety of conditions. Thus, it is common practice in protein chemistry to store enzymes, soluble receptors, etc. in the presence of substrates, products, or other physiological ligand or analogues. Cashikar and Rao (*M110*) investigated the unfolding of red kidney bean acid phospatase in the presence and absence phosphate, a ligand for this enzyme. SEC analysis of the unfolding of the phosphatase demonstrated a marked shift to higher GuHCl concentrations (2 M increase) to disrupt the structure of the dimeric enzyme when phosphate was present. The formation of a single unfolded dimeric intermediate appeared in the presence of phosphate, whereas in its absence, high-molecular-weight aggregates appeared, suggesting a more complex set of intermediate folding forms.

Several reports of the use of SEC to assist protein refolding from denaturing solutions were described during the review period. Batas and colleagues (*M111*−*M113*) studied this procedure in detail using lysozyme and carbonic anhydrase renaturation from urea solutions and coined the term SEPROS (size-exclusion chromatography-based refolding process). The authors consider that the efficiency of the method for producing refolded protein lies in reducing aggregate formation, due to the reduced interaction between partially folded intermediates. Folding intermediate interaction could be limited by the partitioning of the polypeptides between the SEC stationary phase pores, where aggregation would be sterically hindered, and the bulk mobile phase. It was also pointed out that the SEC refolding process adds the value of resolving aggregates from the refolded product. Gauthier and Patston (*M114*) observed a similar advantage in the use of SEC-assisted refolding for recovering active monomeric plasma C1-inhibitor proteinase inhibitor from its inactive aggregated form. These investigators compared the recovery of monomeric C1-inhibitor by SEC, using SDS, GuHCl, and urea to denature the inactivated aggregated protein, obtaining the best recovery from GuHCl unfolded preparations. The authors speculate that the rapidity of removing refolding monomers from aggregates that are forming may enhance the recovery of active material. A method for using SEC-assisted refolding with the use of a cellulosic rolled-bed stationary phase was also described (*M115*, *M116*). This approach may have advantages for scaling-up to production level devices, due to favorable transfer kinetics compared to soft-gel SEC column packing materials, while having the potential for higher linear velocities.

(c) Ligand Binding. A variety of studies examined the binding of materials to proteins in biological samples, such as serum. Since most such studies are not targeted to the derivation of physiochemical parameters, they are not included herein.

SEC may be used to study membrane receptor−ligand interactions either by using either detergent-solubilized protein or by constructing soluble recombinant receptor domains. The quantity of the Na^+/K^+-ATPase pump present in detergent extracts of cells and tissues was assayed by resolution of the protein−ligand complex from the radiolabeled ligand; the high-affinity inhibitor ouabain was successfully employed (*M117*). Analysis of the binding of recombinant human growth hormone (rhGH) to its receptor offers an alternative to the use of animal testing for a potency assay (*M118*, *M119*). The determination of rhGH occurs by binding the ligand to a recombinant-soluble extracellular receptor domain, which forms a complex of two receptors per ligand. The receptor complex is readily resolved from excess unbound receptor and other components that may be present. The use of fluorescence detection permits the assay to be conducted at low rhGH concentrations, appropriate for a complex with a dissociation constant in the nanomolar range, providing high sensitivity and selectivity for variant forms of growth hormone. A similar approach was employed to measure the ability of soluble recombinant human erythropoietin (EPO) receptors to bind EPO (*M120*) and to screen for binding ability of randomly mutagenized EPO receptor during refolding (*M121*). SEC permitted detection of unfolded and folded receptors and their complexes with EPO in crude refolding mixtures. The assay was capable of distinguishing permissive from inactivating amino acid substitutions of the receptor.

Sanny and Price (*M122*) described the use of SEC to determine antibody−antigen binding constants. High affinity of the antibody−antigen interaction permits the identification of components resolved by SEC and, since dissociation is slow, ready determination of equilibrium concentrations without the need of additional assumptions to maintain mass balance. The method was illustrated by the analysis of a monoclonal antibody interaction with human serum albumin and of a serum antivenom with diamondback rattlesnake venom. Determination of the stoichiometry of binding both polyclonal and monoclonal anti-Ro antibodies to the Ro autoantigen by SEC employed titration assay as well as estimation of the molecular mass of complexes (*M123*). Low to moderately high affinity binding of antigens to antibodies permits the recovery of antigen-free antibodies by SEC, providing better recovery of the antibody with higher purity and more rapidly than can be obtained by dialysis (*M124*). This approach was shown to be appropriate for systems with association constants in the range of 5×10^2−0.5×10^6 M^{-1}.

A small-scale frontal gel SEC method for analyzing small-molecule binding to proteins was described by Honjo et al. (*M125*). Through the use of small columns (4.6 mm × 50 mm) packed with hydrophilic silica-based SEC packing material and optimized system plumbing, the total volume of required sample was reduced to 1−2 mL. The validity of the method was shown by determination of the binding stoichiometries of warfarin, tryptophan, and flavin mononucleotide to serum albumins and of *o*-nitrophenol to catechol 2,3-dioxygenase.

Binding of the ReLPS lipopolysaccharide (LPS) to LPS-binding protein and the soluble cell surface antigen CD14 was investigated by SEC and gel electrophoesis, supporting a model by which LPSs bind to their binding protein and then subsequently associate with CD14 (*M126*). Modification of human insulin by palmitic acid at a lysine residue (*M127*) was undertaken to promote the association of insulin with serum albumin, with the goal of improving the bioavailability of insulin. SEC analysis was used to demonstrate the specific binding of the lipid-modified insulin to serum albumin. Rissler and Engelmann (*M128*) defined conditions for efficiently radio-iodinating insulin, purified the material by SEC, and employed the material for radioreceptor binding assays. Bach and Rechler (*M129*) compared SEC and charcoal adsorption

methods to quantify affinities and capacities of insulin-like growth factor II (IGF-II) binding to six isolated IGF binding proteins.

INVERSE SEC

Inverse SEC is a method in which a material of interest is packed into a chromatographic column, and test solutes of known molecular weight are injected to probe the porosity or pore size distribution of the packing. Hagel et al. (*N1*) reviewed the use of SEC for determining pore dimensions of chromatographic packings. The authors concluded that, since SEC cannot provide information about the pore structure, the pore size obtained is dependent upon the selected pore model. Jerabek (*N2*) also discussed inverse SEC as a method for morphological characterization and recommended that this approach is of special value for characterizing swollen polymeric materials. Potschka (*N3*) surveyed inverse SEC and related it to universal calibration.

Guan and Guiochon (*N4*) used inverse SEC to measure the external porosity of columns packed with different commercial HPLC reversed-phase media. In a subsequent study, the pore size and surface area distributions of these packings were reported (*N5*). The pore structure of zirconia HPLC packings was examined by Carr and colleagues (*N6*, *N7*) using inverse SEC. This approach was used for evaluating the surface area, pore volume, and pore size distribution of a variety of zirconias and silica−zirconia composites (*N8*). Inverse SEC was used to characterize microcapsule permeability (*N9*), pumice (*N10*), coal extracts (*N11*), cellulosic fibers (*N12*), and wood pulps (*N13*, *N14*).

PREPARATIVE SEC

A book on preparative SEC using Sephadex LH-20 was written by Henke (*A1*). Roy and Nitsche (*O1*) presented the theory and experimental work for performing large-scale size exclusion separations by multistage countercurrent process using controlled pore glass as the packing. Teraoka and co-workers (*O2*−*O5*) described a new technique, high osmotic pressure chromatography (HOPC), for preparative- and process-scale separation of polymers by molecular weight. In HOPC, a highly concentrated solution of polymer is injected into a packed column until the solution fills the column. During elution, the front end of the solution is enriched with high-MW components, and later fractions contain more low-MW material. Finally, preparative SEC has been applied to the fractionation of acetosolv sugar cane bagasse lignin (*O6*), oligosaccharides from polysaccharides (*O7*), and whey proteins (*O8*).

COUPLED COLUMNS/COLUMN SWITCHING

Suortti (*P1*) combined SEC and anion-exchange chromatography for two-dimensional analysis of oligosaccharides and polysaccharides. Ruiz-Calero et al. (*P2*) used a similar approach for the analysis of low-MW heparins. The coupling of SEC and normal-phase HPLC to GC for the analysis of complex hydrocarbon mixtures was described Blomberg et al. (*P3*). Interfacing between columns was achieved with an on-line solvent evaporator. This technique, as well as GC−GC and LC−LC−GC, was reviewed by Schoenmakers et al. (*P4*). An on-line SEC−GC apparatus was reported by Vruels et al. (*P5*) for determining organophosphorus pesticides in olive oil.

FIELD-FLOW FRACTIONATION

During this review period, there has been a significant increase in the use of field-flow fractionation (FFF) for characterizing the size of particles and macromolecules. The most popular FFF subtechnique for particles is sedimentation FFF, and, for macromolecules, thermal and flow FFF methods dominate. In this review, we will restrict coverage mainly to macromolecules or associated structures rather than particles. General reviews of FFF are given in refs *Q1*−*Q7*. The use of multiangle light scattering (MALS) detection for FFF was reviewed by White (*Q8*) and Johann (*Q9*).The *Journal of Liquid Chromatography and Related Technology* devoted a special issue on FFF (*Q10*).

Flow FFF. Wijnhoven et al. (*Q11*) evaluated several types of membranes for use in organic solvents for flow FFF in an asymmetric channel; best results were found with a fluoropolymer membrane. Nguyen and Beckett (*Q12*) developed a calibration method using polydisperse standards. MALS was used as a detector for the characterization of sulfonated polystyrene (*Q13*), pullulans (*Q14*, *Q15*), and dextran (*Q15*). Hassellov et al. (*Q16*) coupled flow FFF to a high-resolution MS with electrospray ionization and tested the method with low-MW sulfonated polystyrenes.

Williams (*Q17*) presented a new design for an asymmetrical flow FFF channel for uniform channel velocity. Moon et al. (*Q18*) utilized a small, permeable frit near the injection point in an asymmetrical flow FFF channel to bypass the focusing/relaxation procedure. Benincasa and Giddings (*Q19*) examined critical issues affecting the separation of polyelectrolytes by flow FFF, such as sample recovery, overloading, and ionic strength effects. Wijnhoven et al. (*Q20*) studied the retention behavior of proteins, pullulan, and sulfonated polystyrene as a function of injected mass and ionic strength using hollow-fiber flow FFF. Li and Giddings (*Q21*) evaluated a modified flow FFF technique termed membrane-selective flow FFF, used for the isolation and size distribution measurement of colloids in human plasma.

Asymmetrical flow FFF was used to characterize polystyrene lattices, sulfonated polystyrene, globular proteins, and poly(1-vinyl-2-pyrrolidone) (*Q22*); humic acids in solution (*Q23*); high-MW proteins present in glutenin (*Q24*); and the aggregation behavior of a charged, amphiphilic graft copolymer prepared from poly(styrene-*co*-methyl methacrylate-*co*-maleic anhydride) and poly(ethylene oxide) monomethyl ether (*Q25*−*Q27*). FFF has also been applied to the characterization of wheat proteins (*Q28*, *Q29*); lipoproteins from human serum (*Q30*); whey proteins, casein micelles, and fat globules in dairy products (*Q31*); colloidal components in reconstituted skim milk (*Q32*); natural dissolved organic matter in seawater (*Q33*), reservoir water (*Q34*), and drinking water (*Q35*); humic materials (*Q36*, *Q37*); and diblock copolymers of polystyrene-*block*-polybutadiene (*Q38*).

Sedimentation FFF. Sedimentation FFF has been used to study the adsorption of macromolecules onto particles, such as the covalent binding of antibodies on latexes (*Q39*), Pluronic F108 on polystyrene nanospheres (*Q40*), poly(*N*-vinyl-2-pyrrolidone) and a hydrocarbon/fluorocarbon surfactant on hydrophilic/hydrophobic silica (*Q41*), IgG on polystyrene latex (*Q42*), orthophosphate on colloidal river water samples (*Q43*, *Q44*), and triblock copolymers on latexes (*Q45*). Other applications of interest are size measurements of pharmaceutical emulsions

(*Q46*), intravenous fat emulsions (*Q47*), and supramolecular structures of amylopectin, amylose, and their derivatives (*Q48*). Hanselmann et al. (*Q49*) coupled sedimentation FFF with on-line MALS for investigating the structural properties of starch and its dependence on dissolution conditions. Diffusional mass transfer (composition ripening) between emulsion droplets using sedimentation FFF was reported by Arlauskas and Weers (*Q50*).

Thermal FFF. A review on thermal FFF with emphasis on industrial polymer characterization was written by Lee (*Q51*). As compared to SEC, thermal FFF is preferred for the MWD measurements of ultrahigh-MW polymers. For polymers of MW lower than 10 000, SEC provides better resolution. In addition, thermal FFF has the added advantage of being able to detect gel particles in polymer samples.

Jeon and Schimpf (*Q52*) reported a two-dimensional separation in which SEC fractions were analyzed by thermal FFF. Thermal diffusion coefficients obtained from thermal FFF retention ratios were used to obtain average chemical compositions of the SEC fractions. This approach was applied to blends and copolymers of polystyrene and poly(ethylene oxide). Schimpf (*Q53*) also combined thermal FFF with a viscosity detector for determining the MW and chemical composition of copolymers.

Venema et al. (*Q54*) coupled thermal FFF with on-line hydrodynamic chromatography. A two-dimensional separation was obtained with respect to size and thermal diffusion, the latter of which can be related to chemical composition. Van Asten et al. measured the thermal diffusion of polybutadiene and polytetrahydrofuran in various solvents (*Q55*) and polystyrene in binary mixtures of THF/dioxane and THF/cyclohexane (*Q56*). They found that these values are independent of MW but strongly influenced by the chemical composition of both solvent and polymer.

Cho et al. (*Q57*) used thermal FFF to measure thermal diffusion coefficients of poly(styrene-*co*-methyl methacrylate) and poly(styrene-*block*-isoprene). A universal type calibration curve, log(wt % $M[\eta]$) vs log(D/D_T), was used for determining MW values. Ko et al. (*Q58*) discussed enhanced MW selectivity in thermal FFF as being caused by the temperature dependence on the ratio of the diffusion coefficient to the thermal diffusion coefficient.

Nguyen and Beckett (*Q59*) described a thermal FFF calibration method which utilizes one or more broad standards of known M_w values. Reschiglian and colleagues (*Q60*) presented a calibration approach based on the ordinary and thermal diffusivities of a polymer. They stressed that proper sample loading and thermal field strength (linearity conditions) must be chosen to obtain accurate data. These authors also reported on the assessment of linear conditions in thermal FFF by peak shape analysis (*Q61*).

Xu and co-workers (*Q62*) investigated the effect of channel orientation on thermal diffusion and polymer retention in thermal FFF. Myers et al. (*Q63*) studied the effect of the cold-wall temperature on retention behavior. Ryoo et al. (*Q64*) described the influence of gravitational effects of polystyrene and PMMA retention behavior in thermal FFF. A new retention model was developed by Martin et al. (*Q65*) which takes into account a linear variation of the analyte−field interaction parameter from the accumulation wall to the depletion wall.

Lee and Kwon (*Q66*) combine thermal FFF with MALS for characterizing ultrahigh-MW polymers used for intraocular lenses. The size and composition of core−shell latexes were determined using flow and thermal FFF techniques, respectively (*Q67*). Thermal FFF was used to isolate the polymeric and rubber particulate components of acrylonitrile−butadiene−styrene plastics (*Q68*). With this approach, the particle size distribution of rubber particles and the MWD of the polymer components were obtained. Methyl methacrylate−styrene linear diblock copolymers were used to investigate the influence of temperature, MW, and chemical composition on their Soret coefficient using thermal FFF (*Q69*).

SELECTED APPLICATIONS

Asphalts, Bitumins, and Fossil Fuels. SEC analysis has been reported for asphaltenes (*R1*), Corbett asphalt fractions (*R2*, *R3*), polymer-modified asphalts (*R4*−*R6*), bitumens (*R7*), and coal liquefaction extracts (*R8*). Herod et al. (*R9*) examined coal extraction efficacy of THF and 1-methyl-2-pyrrolidinone and concluded that high-MW components are usually not observed during SEC because of insolubility, physical entrapment within the SEC column, or low UV absorptivity.

Carbohydrates, Polysaccharides, and Cellulosics. Churns (*R10*) reviewed the use of SEC for carbohydrate separation including molecular-weight-sensitive detectors. SEC methods for guar, carob, and xanthan were surveyed by Runyon et al. (*R11*). Chen et al. (*R12*) investigated the solubility of starch samples with different amylose/amylopectin ratios in NaOH and DMSO solutions. For these experiments, 0.001 N KOH was used for the mobile phase. Bately and Curtin (*R13*) also investigated the influence of the amylose/amylopectin ratio of starches on sample preparation and SEC behavior.

The molecular architecture of green gram (*Phaseolus aureus*) starch fractions was reported using enzymatic methods and SEC (*R14*).

Rammesmayer et al. (*R15*) used both SEC and reversed-phase HPLC for structural analysis of debranched glucans. SEC with postcolumn calcofluor flow injection analysis was used by Izawa et al. (*R16*, *R17*) for the analysis of β-glucan in wort and beer. SEC and calcofluor binding measurements were used by Nischwitz et al. (*R18*) to monitor the MWD change of β-glucan during malting.

Fishman et al. (*R19*) used SEC with multiangle light scattering and viscosity detectors for the characterization of *Pseudomonas exopolysaccharides*. Denuziere et al. (*R20*) applied a statistical skewing factor for examining the MWD of chitosans. Chitosan degradation from hydrolysis, radiolysis, and enzymatic action was studied by Boryniec et al. (*R21*) using SEC. MW measurements of low-MW heparins was accomplished using SEC columns calibrated with a heparinase-degraded heparin standard (*R22*). Kunz et al. (*R23*) analyzed human milk oligosaccharides using Fractogel TSK HW 50S for acidic oligosaccharides, Fractogel TSK HW 40S for neutral oligosaccharides containing <6 monomers, and Bio-Gel P-4 for neutral oligosaccharides containing >6 monomers.

Silva and Laver (*R24*, *R25*) used DMAC containing LiCl for the solubilization of wood pulp cellulose and reported that optimum dissolution conditions depended on the MW, crystallin-

ity, and lignin content of the sample. Picton et al. (*R26*) used SEC, as well as other methods, to characterize hydroxyethyl cellulose, carboxymethyl cellulose, and cellulose ethers. The hydroxypropyl methyl cellulose and poly(propylene glycol) contents in ophthalmic solutions were determined simultaneously by SEC (*R27*). MW degradation of plasticized nitrocellulose was measured with SEC by Bohn and Volk (*R28*).

Humic Acids and Related Compounds. SEC was used to characterize dissolved organic matter in water samples (*R29*−*R32*) and humic-like substances in urban composts (*R33*). Trubetskoj et al. (*R34*, *R35*) used SEC, polyacrylamide gel electrophoresis, and ultrafiltration for the analysis of humic acids. For the SEC mobile phase, either Tris-HCl or 7 M urea was used with Sephadex G-75. Hongve et al. (*R36*) evaluated a silica-based and two polymer-based SEC columns and different aqueous mobile phases for characterizing humic substances. Schmidt et al. (*R37*) used protein calibration for SEC of lignin and humic acids samples.

Inorganic Compounds and Colloids. A review was given by Antipin and Sakodynskii (*R38*) on SEC of organometallic and multifunctional metal-containing compounds. SEC of mixtures of different aluminum species present in antiperspirants was carried out using Lichrosorb reversed-phase columns (*R39*). Multielement speciation of tea infusions was described, using cation-exchange separation and SEC in combination with ICP/MS (*R40*).

Logue et al. (*R41*) described an SEC method for determining the relative MWD of alumina, silica, and hydroxyaluminosilicate colloids using a G3000 PWXL column and pH 5, 0.1 M $NaNO_3$ as the mobile phase. The hydrodynamic sizes of dextran-coated iron oxide nanoparticles were measured with SEC (*R42*).

Lignins and Tannins. Quaternary amine complexes of lignins were analyzed by an ion-pair type SEC method in 20 mM quaternary amine in THF and styrene−divinylbenzene gel columns (*R43*). With this approach, intermolecular association and adsorption on the packing were overcome without sample derivatization. SEC of lignin from kraft pulping was reported (*R44*). MWD of tannins from wood, bark, and leaves was given by Cadahia et al. (*R45*).

Natural Products. SEC was used for the characterization of fats (*R46*−*R48*). SEC was also used for the analysis of total chlorogenic acid, sucrose, trigonelline, and caffeine in green coffee (*R49*).

Sample Cleanup/Pretreatment. Sample preparation using SEC has been reported for residue analysis of pesticides and herbicides in agricultural products (*R50*−*R52*), marine sediments (*R53*), fat-containing foods and biota samples (*R54*), wool wax and lanolin {*R55*); veterinary drugs in foodstuffs (*R56*); metabolites of polychlorinated biphenyls, dibenzo-*p*-dioxins, and dibenzofurans in microsomal assay extracts (*R57*); PAH in airborne particulates (*R58*); and trace organic compounds in environment-related samples (*R59*).

Synthetic Polymers. Selected papers on the application of SEC for polymer characterization have dealt with phenol−formaldehyde resol resins (*R60*), novolac resins (*R61*), unsaturated polyester prepolymers (*R62*), urethane oils (*R63*), polyethylene (*R64*), poly(phenylene sulfide) (*R64*, *R65*), block−graft copolymers (*R66*), nonlinear block copolymers of $A(AB)_2$, $A(BA)_3$, and $(AB)_3A(BA)_3$ types (*R67*), nylon 11 (*R68*), poly(acrylic acid) and poly(methacrylic acid) (*R69*), polybutene (*R70*), and UV stabilizers in PET bottles (*R71*).

Biopolymers. SEC applications for biopolymer analyses have become the province of hydrophilic small-particle packings, with greater than 90% of column applications now using particles of 20-μm diameter or less. Silica-based packings are heavily favored for applications that require higher throughput, e.g., biopharmaceutical QA/QC, stability studies, and clinical analyses, whereas research applications favor the use of polysaccharide-based materials. The trend in both kinds of packing materials has been to smaller particles for increased efficiency and high sample throughput and smaller internal diameter columns for increased mass sensitivity of detection. The use of on-line mass spectral detection, laser light scattering, and fluorescence detection has grown in the past few years.

(a) Proteins and Peptides. SEC remains a mainstay technique in protein chemistry, for both preparative and analytical separations. The rapid growth in the popularity of obtaining proteins by high-level expression in bacterial cells by recombinant DNA techniques, as opposed to extraction and purification from other biological sources, has greatly expanded the available number of proteins of interest. The vast number of reports of the use of SEC for the isolation or characterization of recombinantly expressed proteins precludes listing them all in this review.

A general review of the use of HPLC for protein biotechnology, including examples of the use of analytical SEC, was presented by Janis et al. (*R72*). Several groups reported the development of SEC assays for recombinant protein biopharmaceuticals, including the receptor-binding assay for human growth hormone (*M118*, *M119*; see section on Physiochemical Studies), a method to distinguish high-molecular-weight aggregates, dimers, and monomers of bovine somatotropin (*R73*, *R74*), the measurement of the concentration of acidic fibroblast growth factor in topical formulations (*R75*), and an SEC immunoassay technique to characterize and quantify the levels of aggregated recombinant human factor VIII in formulations containing 1000-fold excess human serum albumin (*R76*).

Development of useful protein biopharmaceuticals requires attention in developing appropriate formulations, due to the potential for both physical and chemical alterations of proteins on storage. SEC methods are generally used for determining the formation of protein oligomers during storage and formulation. Varley and colleagues (*R77*) demonstrated the use of SEC for product development, formulation, and stability studies and compared SEC with analytical ultracentrifugation. The degradation of monomeric recombinant human interleukin-1 receptor antagonist (rhIL-1ra) during storage in aqueous solutions occurred with the formation of a dissociation-resistant dimer (*R78*), which retains bioactivity. The formation of the dimer was reduced by addition of increasing amounts of sucrose.

A number of studies used SEC to study the aggregation of therapeutic proteins prepared in solid formulations, using lyophilization or spray-drying techniques. Yoshioka et al. (*R79*) studied aggregate formation in lyophilized cakes of bovine serum albumin and bovine γ-globulin (BGG) by SEC and solid-state proton NMR, observing that aggregation of the resolubilized proteins was strongly determined by the hydration levels of the lyophilized cake. These studies were extended to determine the

effects of including dextrans of various molecular weights to solutions of BGG before lyophilization (*R80*). Duddu and Dal-Monte (*R81*) determined the aggregation of a therapeutic monoclonal antibody in lyophilized formulations containing sucrose and trehalose as stabilizers. SEC methods were also developed to study the recovery of active interferon-γ throughout the process of encapsulating the protein in polylactic−coglycolic (PLGA) biodegradable microspheres (*R82*) and of active L-asparaginase and superoxide dismutase encapsulated in poly(alkyl cyanoacrylate) particles (*R83*).

Lipoprotein analysis by SEC remains an area of considerable activity, offering the potential advantages of saving time and being amenable to automation. A general review of SEC methods for lipoprotein analyses was presented by Barter (*R84*). Methods for the preparation of lipoproteins by density ultracentrifugation and SEC were compared, and it was observed that the SEC retention of apolipoproteins E and A-I is sensitive to both the ionic strength and the pH of mobile phases (*R85*). Appropriate mobile phase conditions were developed to improve the performance of the SEC separation. Use of SEC methods in the clinical setting and comparison with established techniques were reported for low-density lipoprotein (LDL) serum analyses (*R86*) and for the determination both low- and high-density lipoprotein (HDL) cholesterol (*R87*). Since glycated lipoproteins, but not nonglycated lipoproteins, bind to a boronate affinity column, Tanaka et al. (*R88*) were able to employ column-switching to resolve glycated and nonglycated lipoproteins in-line with SEC analysis of LDLs and HDLs.

Separations of synthetic and biologically active peptides by SEC for preparative and analytical purposes continue to be widely reported. Separation conditions and examples of the use of SEC for analysis of synthetic peptides were reviewed by Mant and Hodges (*R89*) and for neuropeptides by Irvine (*R90*). An SEC method for the analysis and isolation of tandem-repeat peptides with intact synthetic protecting groups used dimethyl formamide as the mobile phase (*R91*). The processing of probrain natriuretic peptide in blood, serum, and plasma was investigated by SEC with immunoassay (*R92*). Analysis of peptides generated by peptic hydrolysis of hemoglobin and of myoglobin was aided by the use of diode array detection, with second-order derivative spectral analysis (*R93*). Direct analysis of the LVV-hemorphin-7 peptide content of cerebrospinal fluid samples by SEC with off-line ESI-MS was presented by Silberring and Nyberg (*R94*).

(b) Nucleic Acids. In comparison to protein chemical applications, there was limited growth in the use of SEC for nucleic acid separations during the review period. The protective effects of BSA in various salt and buffer solutions on DNA damage induced by ionizing radiation were demonstrated by SEC analysis of HMW DNA (*R95*). Plasmid DNA was purified by SEC using various column packing materials, including HEMA (*R96*), Superose, and Sephacryl (*R97*), suggesting SEC as an efficient alternative to density gradient ultracentrifugation techniques. Cole (*R98*) used axially applied electric fields to effect changes in the elution of DNA fragments undergoing separation on SEC columns. The effects of altering packing material, field strength, and flow rate were evaluated. Hirabayashi and Kasai (*R99*) continued their studies of the separation of double-stranded DNAs by the so-called "slalom chromatography" technique. The authors found that separations could be conducted on small particle diameter reversed-phase column packing materials, and retention could be modified by flow rate and mobile phase ionic strength.

(c) Complexes. Improved functional reconstitution of the bovine brain benzodiazapine receptor into lipid vesicles was accomplished by SEC, which depleted the protein−lipid-detergent mixtures of the membrane-solubilizing detergent component (*R100*). The resulting proteoliposomes exhibited an increased number of operative binding sites and remained stable in storage. SEC methods were developed to measure the associations of several synthetic anionic polymers with cationic liposomes (*R101*) and of the surfactant, sucrose ester P-1670, with milk protein micelles (*R102*). The associations of peptides with micelles of SDS, sodium decanesulfonate, and a nonionic detergent were quantitatively investigated using micellar liquid chromatography with a SEC column (*R103*). Analysis of fractions obtained by preparative SEC separations of hepatitis B surface antigen particles showed unanticipated variability in size (*R104*).

(d) Polysaccharides/Proteoglycans. Structure determination of glycoprotein oligosaccharides by combined enzymatic digestion and SEC analysis was reviewed by Prime et al. (*R105*). Primary structure analysis of peptidoglycan trimers from murein was achieved by a combination of SEC, reversed-phase HPLC, and mass spectrometry (*R106*). A method for the purification and molecular weight estimation of disaggregated intact proteoglycans from rat chondrosarcoma cells was described (*R107*). SEC was employed to isolate and characterize the presence of aggrecan in human sclera tissue (*R108*).

SEC methods to investigate the MWD of hyaluronans were presented and conditions for manipulating samples described (*R109*). Since hyaluronan preparations are employed during opthalmic surgery, Equi et al. (*R110*) used SEC to measure the MWD of enzymatically digested sodium hyaluronans to define the effects of molecular weight on intraocular pressure following injections into the eye. SEC-LALLS and viscometry measurements of hyaluronans showed elevated concentrations in synovial fluid samples from patients with degenerative joint disease and diabetic arthropathy (*R111*). Procedures for tissue processing and sample preparation and storage, as well as recovery experiments, were described for the SEC determination of FITC-labeled dextrans (*R112*) and dextrans and pullulans (*R113*).

(e) Others. The use of coupled columns to isolate components of interest from the bulk of impurities can greatly simplify measurement of analytes present in complex sample matrixes. For the analysis of voriconazole, an antifungal agent, Stopher and Gage (*R114*) used a first-dimension SEC column to isolate selected components of human plasma for resolution and quantification by a second-dimension reversed-phase HPLC separation. Similarly, Amari and Mazaroff (*R115*) partially resolved recombinant interleukin-11 from total cellular components of *E. coli* by SEC and then obtained resolution of the protein by second-dimension reversed-phase HPLC.

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