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Determination of biopolymer (protein) molecular weights by gradient elution, reversed-phase high-performance liquid chromatography with low-angle laser light scattering detection

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SUMMARY

The determination of molecular weights for certain proteins has been performed. This has involved the on-line coupling of gradient elution, reversed-phase high-performance liquid chromatography (RP-HPLC) with low-angle laser light scattering (LALLS) detection. A new 1.5-μm, non-porous, Monosphere RP-C_8 column has been used in order to perform fast and conventional RP-HPLC gradients (5–45 min). Traditional specific refractive index increment (dn/dc) and refractive index (n) measurements have been performed in order to derive absolute weight-average molecular weight ($\bar{M}_w$) information for ribonuclease A, lysozyme, and bovine serum albumin. Standard mixtures of known concentrations of each protein have been separated using reversed-phase gradients utilizing acetonitrile with on-line LALLS determination of excess Rayleigh scattering factors. Accurate $\bar{M}_w$ data have been obtained for all three proteins, but only under certain, conventional reversed-phase gradient elution conditions. Between 5–10 min of fast gradient elution, each protein appears to exhibit unusual $\bar{M}_w$ values, suggestive of aggregate formations. Methods have been developed to define the nature of such aggregates. The on-line coupling of modern RP-HPLC for biopolymers with LALLS represents a major step forward in the ability of bioanalytical chemists to determine the nature (monomer *versus* aggregate) of such materials. Other classes of biopolymers should prove suitable for studies with the same RP-HPLC–LALLS–UV approaches.

INTRODUCTION

The separation and determination of biopolymers by modern high-performance liquid chromatography (HPLC) has become an area of intense interest within the past few years[1–5]. Separation approaches for biopolymers have advanced rapidly,

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however, methods of detection have lagged somewhat behind these advances[6,7]. Most of the routine detectors for HPLC, such as ultraviolet–visible (UV–VIS), fluorescence (FL), differential refractive index (DRI), and electrochemistry, offer little in the way of biopolymer information or characterization. Most biopolymers show little or no response under conventional electrochemical detection (ED) conditions, in the absence of some type of derivatization. Though UV–FL detectors may provide some information, they rarely provide biopolymer identification or characterization. This is because in UV–FL detection, most biopolymers have very similar absorbance and emission spectra. Thus, in the past, chromatographic performance via capacity factor determination remains the most widely used identification parameter.

We have recently described the use of low-angle laser light scattering (LALLS) with linear diode array (LDA) detection coupled to hydrophobic interaction chromatography (HIC) for alkaline phosphatase enzymes[8] and have had even more success with other chromatographic systems. Even with just the LDA's complete spectral data profile, it remains difficult to identify/characterize proteins or their aggregates without the use of LALLS for $\bar{M}_w$ determination.

LALLS has been utilized in the past for various biopolymers in HPLC, but it has generally been restricted to isocratic conditions, mainly via size exclusion chromatography (SEC)[9–14]. It has rarely been utilized in combination with mobile phase gradient elution conditions[9]. It was our interest to investigate the use of modern chromatographic methods for protein characterization involving the use of solvent gradients. For example, HIC utilizes salt gradient elution conditions for biopolymers[15–19] (ref. 19 contains a special section on HIC, together with excellent reviews). We have recently demonstrated the advantages possible in using gradient elution HIC conditions together with LALLS for biopolymer characterization[8–10].

The use of aqueous–organic gradients in reversed-phase (RP)-HPLC has been widely described and applied in the literature, often showing high resolving capabilities, but usually resulting in the loss of enzymatic activity[20–26]. Conventional gradient elution RP-HPLC for proteins/enzymes has used methanol, acetonitrile, and isopropanol as general organic modifiers. Most RP-HPLC for biopolymers has employed 5–10-μm, wide-pore (*ca.* 300 Å), C_8 or C_{18} bonded phases, of conventional column dimensions (10–25 cm × 2.0–4.6 mm I.D.). There have been descriptions of 3-μm RP supports being used, and this can be one type of approach for fast-RP gradient elution of biopolymers[27–28]. Unger and co-workers[29–34] have recently described a totally non-porous, monodisperse, 1.5-μm bonded silica used in the separation of proteins by HIC and RP-HPLC. Essentially, the pore size here is related to the surface area. Their studies have involved both 1.5-μm HIC, as well as RP (C_8) bonded silica supports for fast gradient elution biopolymer separations. Horváth's group has also published extensively in the area of fast gradient RP-HPLC for proteins[28,35,36]. In addition, several industrial firms have now commercialized special columns for the fast elution of biopolymers, with emphasis on proteins[37].

There is no discussion in the literature wherein any type of aqueous–organic, RP gradient elution separation of biopolymers was coupled in series with on-line LALLS measurements[9]. There have been suggestions that this would be impractical, mainly because of changes in mobile phase refractive index (n) and changes in the specific refractive index increment (dn/dc) of the eluting biopolymer. Such changes were expected to invalidate any on-line LALLS measurements. We have been interested in

demonstrating the ability of using most modern biopolymer HPLC separations with on-line LALLS measurements. Our initial success with salt gradient HIC–LALLS and isocratic SEC–LALLS for bovine alkaline phosphatase[8] and β-lactoglobulin A (β-lact A)[10], led us to consider certain gradient elution RP-HPLC–LALLS-UV methods for other biopolymer systems (*i.e.*, proteins and enzymes).

This paper describes the coupling of RP-HPLC with on-line LALLS–UV measurements while using traditional dn/dc and n determinations with standard computer data acquisition for weight-average molecular weight ($\bar{M}_w$) computations. Gradient elutions were performed with varying ratios of water–acetonitrile over times from 5–45 min, using linear gradients. Efforts have been made to understand the limitations of fast RP-HPLC–LALLS–UV approaches for proteins/enzymes, as well as its advantages and possibilities. Ribonuclease A (RNase A), lysozyme (LYS), and bovine serum albumin (BSA) were the proteins investigated. Results have suggested that this approach should be amenable to many other proteins/enzymes, and conceivably to many other classes of biopolymers.

THEORY

The design of the KMX-6 LALLS photometer differs considerably from that of conventional light scattering photometers. In brief, the red light from a He–Ne laser at 632.8 nm is focused onto the sample cell and solution contained between two silica windows. The light scattered at low-angles, namely 6–7°, determined by a series of annuli and known solvent refractive index, is detected by a photomultiplier tube. The measured quantity is the ratio of the scattered to the transmitted radiant power. Sample and scattering volumes are small, 10 μl and 35 nl, respectively, using the flow through cell. The calibration method is based on geometry, hence the resulting measurements are absolute rather than being referenced to a known scattering standard. The reader is referred to references 8–10, 38–51 and others for a more in-depth review.

The calculated Rayleigh factor of solutions using the Chromatix KMX-6 is simply the quantity

$$R_\theta = \frac{G_\theta}{G_0}(D)(\sigma' l')^{-1} \tag{1}$$

where G_θ = intensity of light scattered from the solution at an angle θ, D = transmittance of the attenuators used in measuring the incident illuminating light beam, G_0 = illuminating light beam transmitted through the sample at the incident angle, and the product $(\sigma' l')^{-1}$ is a function of solution refractive index n', for the available annuli, recommended field stops and cell type. The average of median scattering angle, $\bar{\theta}$, is also constant. The "excess" Rayleigh factor, $\bar{R}_\theta$, is the difference between R_θ for the polymer solution and that for the solvent.

The equation used for the calculation of $\bar{M}_w$ is:

$$\frac{Kc}{\bar{R}_\theta} = \frac{1}{\bar{M}_w} + 2A_2 c \tag{2}$$

where c is the concentration in g/ml, A_2 is the second virial coefficient, $\bar{R}_\theta$ is the excess Rayleigh factor for solvent, and K is the polymer optical constant (for vertically polarized light) where

$$K = \frac{2\pi^2 n^2}{\lambda^4 N}(\mathrm{d}n/\mathrm{d}c)^2 \tag{3}$$

where n is the solvent refractive index, λ is the wavelength, N is Avogadro's number, and $\mathrm{d}n/\mathrm{d}c$ is the change in n per change in concentration or specific refractive index increment. n and $\mathrm{d}n/\mathrm{d}c$ should be values obtained at wavelength λ.

With monochromatic, vertically polarized light (He–Ne laser, 632.8 nm), eqn. 3 reduces to:

$$K = 4.079 \cdot 10^{-6}(n)^2(\mathrm{d}n/\mathrm{d}c)^2 \tag{4}$$

To measure molecular weight one simply requires measuring $\bar{R}_\theta$ at several different concentrations and then extrapolating the function $Kc/\bar{R}_\theta$ to zero concentration. The intercept results in the reciprocal of $\bar{M}_w$, and the second virial coefficient, A_2, may be determined from one half the slope, in ml mol/g^2. This is deduced from eqn. 2.

In the on-line mode of operation, in conjunction with an HPLC or SEC, one may determine the weight-average molecular weight for each incremental elution point across the peak by applying eqn. 2 in the following form:

$$\frac{K_i c_i}{\bar{R}_{\theta_i}} = \frac{1}{\bar{M}_{w_i}} + 2A_2 c_i \tag{5}$$

Noting that for isocratic HPLC operation analyzing homopolymers, K is constant; however, for heteropolymers or in solvent gradient operation, K will change in addition to the solid angle, $(\sigma' l')^{-1}$.

Eqn. 2 is strictly valid for two-component systems[42–50]; however, aqueous protein solutions generally contain at least three components: water (component 1), protein (component 2), and electrolyte/organic modifier (component 3), and in most cases, more than three components, since a buffer is used to control pH. Therefore, the presence of extra components limits the validity of eqn. 2. This point has been extensively discussed in the literature, and the most important conclusions with regard to the validity of eqn. 2 are: first, the value obtained by extrapolation of eqn. 2 to zero protein concentration will be an apparent molecular weight, $\bar{M}_{w_{app}}$, which contains the product of the molecular weight, $\bar{M}_w$, with a compositional parameter α, and a parameter describing thermodynamic interactions between components 2 and 3, Ψ, *i.e.*:

$$\bar{M}_{w_{app}} = \bar{M}_w(1 + \alpha\Psi) \tag{6}$$

Second, the three component system can be reduced to a two component system if the measurement of the specific refractive index increment, $\mathrm{d}n/\mathrm{d}c$, is performed using the dialyzed polymer solution. Thirdly, under certain experimental conditions, it is

possible to apply eqn. 6 and obtain correct values of molecular weight from the extrapolated value, since the values of the compositional parameter α and/or the interaction parameter Ψ are zero or compensate one another. For example, Edsall *et al.*[49] have shown that for dilute electrolyte solutions and high net charge on the protein, the intercept $Kc/\bar{R}_\theta$ *versus* c can be identified with the inverse of the actual molecular weight. However, the slope is greatly affected by the salt concentration.

Gradient elution in HPLC has characteristics that are very stringent for LALLS measurements: there is a constant change in mobile phase composition, the system is a multiple component system, and the detection of the analyte is performed under dynamic conditions. Therefore, it is necessary to investigate the validity of the method under these conditions.

For a three-component system, the relationship between the specific refractive index increment measured under conditions of osmotic equilibrium, $(dn/dc_2)^0_\mu$, and the conventional specific refractive index increment, dn/dc_2 (formerly noted as simply dn/dc), in the limit of low concentration of protein, c_2, is given by[45]:

$$(dn/dc_2)^0_\mu \approx (dn/dc_2) + (dn/dc_3)_{m_2=0}(1 - c_3V_3)\Upsilon_3 \tag{7}$$

where $(dn/dc_3)_{m_2=0}$ is the specific refractive index increment for the electrolyte/organic modifier solution when the concentration of the protein equals zero, c_3 is the concentration of the electrolyte/organic modifier, V_3 is the specific partial volume of the electrolyte/organic modifier, and Υ_3 is the specific interaction parameter, or selective sorption, of the electrolyte/organic modifier with the protein.

From eqn. 7, it is clear that the left side will be equal to dn/dc_2 when either $(dn/dc_3)_{m_2=0}$, $(1-c_3V_3)$, or Υ_3 is near or equal to zero. Therefore, three-component theory can be artificially reduced and manipulated as a two-component system.

This refractive index increment correction becomes less important with diminishing absolute value of the difference of refractive indices of the two solvent components (n_1-n_3), and becomes zero when they are "isorefractive" $(n_1 = n_3)$. When $n_1 = n_3$, the refractive index of the mixed solvent (components 1 and 3 combined) virtually does not change with its composition. Also, when both refractive increments of the macromolecule, $(dn/dc_2)^0_\mu$ and dn/dc_2 are equal, both molar masses, $\bar{M}_w$ and $\bar{M}_{w_{app}}$ assume the same value. In mixtures of two strictly isorefractive solvents, the true molar mass of a macromolecule is measured by light scattering, regardless of the composition of the mixture and of the extent of selective sorption. Therefore, isorefractive solvent systems can be treated in light scattering measurements simply as single solvents using two-component manipulations.

Isorefractivity may now be defined from the viewpoint of the light scattering method (the method itself has about a $\pm 5\%$ accuracy). $\bar{M}_{w_{app}}/\bar{M}_w$ should therefore be less than 1.05 and the relationship (eqn. 8) must hold:

$$(n_1 - n_3) < (0.025)(dn/dc_2)(1/\Upsilon_3) \tag{8}$$

For a system with a typical value of $dn/dc_2 = 0.15$ ml/g and a small $\Upsilon_3 = 0.10$ ml/g, the solvent components can be regarded as isorefractive if the absolute value of $n_1 - n_3 < 0.038$. In a system with strong selective sorption, $\Upsilon_3 = 1.0$ ml/g and equivalent dn/dc_2, solvents are considered isorefractive when the absolute value of $n_1 - n_3 < 0.0038$, which is much more restrictive.

EXPERIMENTAL

Apparatus

The HPLC–UV system was modular in design, and consisted of a Rheodyne (Cotati, CA, U.S.A.) Model 7125 syringe loading injector fitted with a 20-μl loop, two Waters (Waters-Millipore, Milford, MA, U.S.A.) Model M501 high-pressure solvent delivery systems, a Waters Model 660 solvent programmer, an Altex-Hitachi (Rainin, Berkeley, CA, U.S.A.) Model 100-40 variable-wavelength UV–VIS detector, and a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 3380A reporting integrator. The RP column used throughout this research was a 1.5-μm, Monosphere RP-C_8, non-porous material, and had column dimensions of 3.6 cm × 8 mm I.D. (Merck, Darmstadt, F.R.G., c/o EM Science, Cherry Hill, NJ, U.S.A.).

The RP-HPLC–LALLS–UV, SEC–LALLS–UV–DRI, and FIA–LALLS (a modernized instrumental derivation of the traditional static light scattering experiment utilizing flow injection analysis for sample introduction purposes) systems consisted of two modular arrangements.

System I, used for RP-HPLC–LALLS studies performed at ambient temperature (25°C), 4°C, and −5°C, was composed of an LDC Model CM4000 multiple-solvent delivery system, a Rheodyne Model 7125 syringe loading injector equipped with a 20-μl loop, a Chromatix (LDC Analytical/Thermo Instruments, Riviera Beach, FL, U.S.A.) Model KMX-6 LALLS detector set-up for flow analyses, an LDC SM4000 programmable UV–VIS detector, linked to a Soltec (Sun Valley, CA, U.S.A.) Model 1242 chart recorder, and to an IBM PC-AT compatible computer using PCLALLS (LDC Analytical) data software system.

System II, used primarily for SEC–LALLS–UV–DRI, consisted of an LDC ConstaMetric III analytical metering pump (1/3 speed), a Rheodyne Model 7125 injection valve equipped with a 100-μl loop, a TSK SW-3000 size-exclusion column, 30 cm × 8 mm I.D. (Phenomenex, Ranchos Palos Verdes, CA, U.S.A.), a Chromatix KMX-6 LALLS set-up for flow analyses, an LDC SpectroMonitor-D variable UV–VIS and RefractoMonitor IV DRI detector, all linked to both a Soltec Model 1242 chart recorder and an LDC CMX-10A analog-to-digital (A/D) converter. These A/D converters were linked to a DEC (Digital Equipment, Boston, MA, U.S.A.) Micro PDP-11/23+ computer system for digitization of instrumental analog outputs and further graphics/data manipulation. Software for calculating LALLS molecular weight information was from Chromatix, version MOLWT3.

System II was sometimes configured for FIA–LALLS analyses merely by decreasing the flow-rate to a nominal 0.1 ml/min, increasing the injection loop volume to 1.00 ml, removing the chromatographic column, and having flow directly into the KMX-6 LALLS photometer. The analog output from the LALLS photometer was sent to a Soltec chart recorder and the excess Rayleigh scattering factor was then determined for a series of individual biopolymer concentrations.

The dn/dc determinations were performed in bulk solution, with an off-line Chromatix model KMX-16 laser (633 nm) differential refractometer. Samples were analyzed via conventional methods, which theory permits. Protein concentrations were selected in order to produce data that is well within the linear range of the instrument's capabilities, typically 3–5 mg/ml, while keeping minimal sample consumption in mind.

Mobile phases

Two mobile phases were prepared for gradient elution RP-HPLC–LALLS–UV studies. The first consisted of 0.15% trifluoroacetic acid in water (A), and the second consisted of 0.15% trifluoroacetic acid in acetonitrile–water (95:5) (B). Both solvents were adjusted to pH 3.0 with ammonium hydroxide, degassed under vacuum, and filtered through a 0.2-μm hydrophilic Durapore membrane (Millipore, Bedford, MA, U.S.A.). The column was equilibrated overnight, when not in use, with B–A (50:50) at a flow-rate of 0.2 ml/min. Accurate and precise proportions of these two solvent combinations were used in the FIA–LALLS determinations. The modified protein buffer used for the SEC and FIA–LALLS studies consisted of: 18 m*M* HEPES, 7 m*M* imidazole, 1 m*M* EDTA, 3 m*M* sodium azide, 200 m*M* sodium acetate, 0.5 m*M* non-ionic surfactant, octaethylene glycol-mono-N-dodecyl ether, (Nikko, Tokyo, Japan, part No. NIKKOL BL-85Y), refractive index (RI) at 25°C, 633 nm = 1.3355 refractive index units, pH = 7.0.

Chemicals and supplies

Acetonitrile, methanol and water were HPLC Omnisolv grade, trifluoroacetic acid and ammonium acetate were reagent grade, all from EM Science. All solvents were filtered through a 0.2-μm hydrophilic Durapore filter (Millipore). Protein standards, *viz.*, RNase A, LYS, and BSA, were obtained from Sigma (St. Louis, MO, U.S.A.). All proteins were used as received, without further purification.

Procedures

RP–HPLC–LALLS–UV studies. Standard protein mixtures were prepared in mobile phase A at concentrations in the range of 3–12 mg/ml. The mobile phase composition for actual separation/elution of proteins is given in Results and Discussion. All gradient conditions were held constant except for time. Gradient conditions were 1.6 ml/min, 20–55% B (linear) and was used throughout, generating a back pressure of about 2000–2500 p.s.i. For clarification purposes, fast gradients are herein defined as having a solvent change of greater than 2% B/min, while conventional gradients are less than 2% B/min.

One important point that must be considered before performing LALLS analyses was true injected sample mass. A major source of systematic error has been due to inaccurate, but precise, injection volumes claimed by the manufacturers. Each loop was carefully custom fitted onto a particular injector to ensure a minimum dead volume, if any, between the loop itself and the injector assembly. Its volume was gravimetrically determined and contained the specific volume based on the density of water at the experimental temperature (rearrange $\rho_T = m/V$ to $V = m/\rho_T$). One can then calculate true loop volume to within 2% of actual total volume when connected to the injector. To ensure chromatographic reproducibility, each injection was performed at least five times. Linear gradients ranging from 5–45 min were employed, with simultaneous LALLS and UV detection for the RP-HPLC–LALLS–UV studies.

FIA–LALLS studies. FIA–LALLS determinations used the same mobile phase composition (%B–%A) observed at the point of elution (peak apex conditions) for each protein under RP-HPLC–LALLS–UV. These studies were performed under pure isocratic conditions, and measured bulk solution Rayleigh factors. Concentrations of proteins varied, but were usually in the range of 0.20–6.00 mg/ml.

SEC–LALLS–UV–DRI studies. This conventional approach to $\bar{M}_w$ determination for proteins and other biopolymers utilized a bonded diol phase TSK SW-3000 SEC column at constant flow-rate of 0.75 ml/min, using the modified protein buffer as the mobile phase. UV detection was at 280 nm. Each protein was injected individually, chromatographed at least in triplicate, and all data are given as the mean ± standard deviation (S.D.).

Refractive index (n) and specific refractive index increment (dn/dc) determinations. The refractive index and specific refractive index increment determinations were conducted using a modified Abbé refractometer (Milton Roy, Rochester, NY, U.S.A.) using a 632.8-nm narrow bandpass filter (Melles Griot, Rochester, NY, U.S.A.) between the light source and the receiving optics, and the Chromatix KMX-16, a differential laser refractometer. Experimental determinations of n coincided closely with interpolated literature values. Refractive index measurements, with varying percentages of B, were performed in an off-line study over the gradient range of B used in the RP-HPLC separations. Solutions of 0–100% B were prepared volumetrically in final volumes of 10 ml, with the % B increasing successively by 10 in each solution. The refractive indices of these solutions were determined in triplicate, using standard operating procedures provided with the refractometer and described in the manual.

The dn/dc values for each protein were calculated using the laser differential refractometer, Chromatix Model KMX-16, connected to a low-temperature (20-l) water bath (Fisher Scientific, Boston, MA, U.S.A.). The sample cell of the refractometer was frequently cleaned by flushing with water and then methanol, and dried by suction. The system was allowed to equilibrate for at least 30 min between measurements. The cell compartment temperature was set for 25, 4 or −5°C depending on the required temperature and was allowed to equilibrate for at least 30 min prior to taking actual measurements. To check for cell inconsistencies, readings of air *vs.* air (*i.e.*, an empty cell) were recorded. A reading of about ±30 counts indicated that the cell was clean and the system was operating properly. This procedure was followed for each solution.

The mobile phase composition for each eluting protein was calculated in the customary manner incorporating factors such as gradient lag volume, individual peak retention volume/time, flow-rate, gradient change (%B/min), and initial gradient %B composition.

Protein solutions were prepared in the predetermined mobile phase composition. Concentrations ranged from 3–12 mg/ml. The protein was first dissolved in mobile phase A. Once the solutions were homogeneous and at equilibrium, approximately one hour later, appropriate amounts of solvent B were added. These solutions were mildly swirled for about 10 min and then refrigerated at 4°C overnight. Blanks were prepared identically, side by side.

These sample solutions were then placed into the cell assembly and 6–8 readings were recorded for each sample. The refractive index was measured separately for the blank solution. Subsequent solutions, of different concentrations of proteins and blanks, were analyzed in the same manner.

Orthogonal chromatography (RP–RP) of protein mixtures. Rather than perform reinjection experiments where after initial chromatographic separation an individual peak is collected and reinjected into the same system (resulting in even further dilution), orthogonal chromatography was used using two independent chromato-

graphic systems. The first HPLC system employed a 5-min gradient while the second was running a 20-min gradient. The second injector (Rheodyne model 7010) was modified into a switching valve with a 2-ml loop, which allowed efficient and complete transfer of the analyte of interest from one system to the other under dynamic conditions (Fig. 3). The 2-ml loop was used since the elution volume of each peak from the first system was calculated to be just less than 2 ml.

Low-temperature gradient elution RP-HPLC–LALLS–UV. Low-temperature gradient elution RP-HPLC–LALLS–UV was performed under the same identical gradient conditions as at ambient temperature, except that the column and transfer tubings were equilibrated and maintained in an ice–water bath (4°C) or an ice–methanol bath (−5°C).

RESULTS AND DISCUSSION

This study has been designed to understand various phenomena regarding protein separations under normal ($\geq$15 min) and fast (<15 min) gradient elution conditions. This has involved: (1) determination of chromatographic performance criteria with UV detection; (2) determination of $\bar{M}_w$ for each protein as a function of the gradient employed; (3) determination of aggregation, if any, as a function of the gradient; and (4) overall reproducibility, accuracy, and precision of on-line LALLS determinations under certain gradient RP conditions for some proteins. These are the first studies of any protein eluted under gradient RP conditions monitored by on-line LALLS photometry for determination of $\bar{M}_w$.

We believe that other reports of gradient elution, RP separations of proteins, using conventional detection such as UV, DRI, FL, etc., may have never determined, with a high degree of certainty, the precise nature of the eluting species. Though RP-HPLC coupled to mass spectrometry (RP-HPLC–MS) may have been able to identify proteins, it is not clear that aggregates would survive the MS measurement conditions. There are no obvious reports of aggregate determination via RP-HPLC–MS approaches. The results reported here suggest that proteins behave very differently than most investigators have assumed under RP-HPLC conditions, and that their behavior is a function of the nature of the gradient generated, especially with respect to its speed of formation (change in %B/min).

In order for HPLC–LALLS measurements employing three-component systems (see Theory) to be manipulated as a two-component system, eqn. 7[45] or 8[44] need strict consideration. In RP-HPLC using acetonitrile, the refractive index (at 25°C and 633 nm) for water and acetonitrile at pH 3.0 was 1.332 and 1.341, respectively. Their difference being 0.009 refractive index units, well below the 0.025 boundary of eqn. 8. Protein elution occurred in the region where the refractive index of the buffer combination was linear when plotted *versus* % composition (25–45% B). In summary, for gradient elution to be successfully coupled to LALLS for valid molecular weight determinations, the "isorefractivity" issue must be satisfied.

We present several related studies, including: (1) RP–LALLS–UV chromatograms and $\bar{M}_w$ values for three standard proteins (RNase A, LYS, and BSA), using water–acetonitrile linear gradients with a 1.5-μm Monosphere, C_8, non-porous column, eluting within various times (5–45 min); (2) n and dn/dc values for each protein in the mobile phase composition causing elution under RP-HPLC–LALLS–

UV conditions; (3) orthogonal chromatography of 5-min protein peaks introduced into a 20-min gradient for $\bar{M}_w$ determination; (4) low temperature and concentration studies in RP-HPLC–LALLS–UV with the determination of $\bar{M}_w$ under fast gradient elution RP conditions (10 min); (5) SEC–LALLS–UV–DRI determinations of $\bar{M}_w$ with a modified protein buffer; and (6) FIA–LALLS determinations of $\bar{M}_w$ and A_2 using combinations of water–acetonitrile determined from the RP-HPLC–LALLS–UV experiments. These results demonstrate significant aggregate formation, as a function of the gradient formation times. The overall determined experimental errors, expressed in percent relative standard deviation (%R.S.D.), for all reported $\bar{M}_w$ values, were less than 10%, often less than 5% for SEC–LALLS determinations.

RP-HPLC–UV chromatographic performance criteria for typical proteins using water–acetonitrile linear gradients with a 1.5-μm monosphere, C_8, non-porous column

Others have discussed chromatographic performance with respect to proteins using gradient elution RP conditions with a 1.5-μm, non-porous C_8 silica based column[29–34]. In general, peak shapes, capacity factors, efficiencies, resolutions, plate heights, and asymmetries have been better than what has been possible using conventional ($\geq$3-μm) RP columns under similar gradient elution conditions. Our results are in general agreement with those already reported by Horváth and co-workers[28,35,36] and Unger and co-workers[29–34] using different approaches to the preparation of their stationary phases.

Table I summarizes our data obtained for RNase A, LYS, and BSA under typical fast-RP (5 min) conditions, using the mobile phase indicated (Experimental). We initially chose a narrow gradient profile for these first studies, namely 28–40% B in A, but later work showed that larger gradient ranges were practical. We chose water–acetonitrile in view of their popularity and similar refractive indices, with the expectation that n would not deviate markedly over the range of solvent combinations desired[42–45]. Table I illustrates reproducibility over time. Other column performance

TABLE I

SUMMARY OF RP-HPLC–LALLS–UV MOLECULAR WEIGHT DETERMINATIONS FOR RIBONUCLEASE A, LYSOZYME AND BOVINE SERUM ALBUMIN AS A FUNCTION OF GRADIENT FORMATION TIMES

Gradient time (min)	*Molecular weight ± standard deviation*[a]		
	Ribonuclease A[b]	*Lysozyme*[b]	*Bovine serum albumin*[b]
5	21 700 ± 700	25 600 ± 1100	249 000 ± 12 600
10	18 400 ± 1600	19 800 ± 1000	152 000 ± 14 400
15	14 400 ± 800	15 400 ± 1000	92 400 ± 1300
20	14 500 ± 1300	13 800 ± 1300	93 500 ± 3900
30	15 000 ± 900	14 900 ± 1100	109 500 ± 7300
45	14 200 ± 900	15 700 ± 900	96 700 ± 6100

[a] Standard deviation using 5 measurements throughout, except 30 and 45 min, where 3 measurements were used.

[b] Literature monomer $\bar{M}_w$ values for RNase A = 13 700, LYS = 14 400, and BSA = 65 000 daltons.

criteria such as capacity factor, theoretical plates, resolutions, plate heights and asymmetries have been determined, but not shown here, and are in agreement with Unger and co-workers[29–34]. In general, there is excellent reproducibility and precision, as indicated by the low %R.S.D. values.

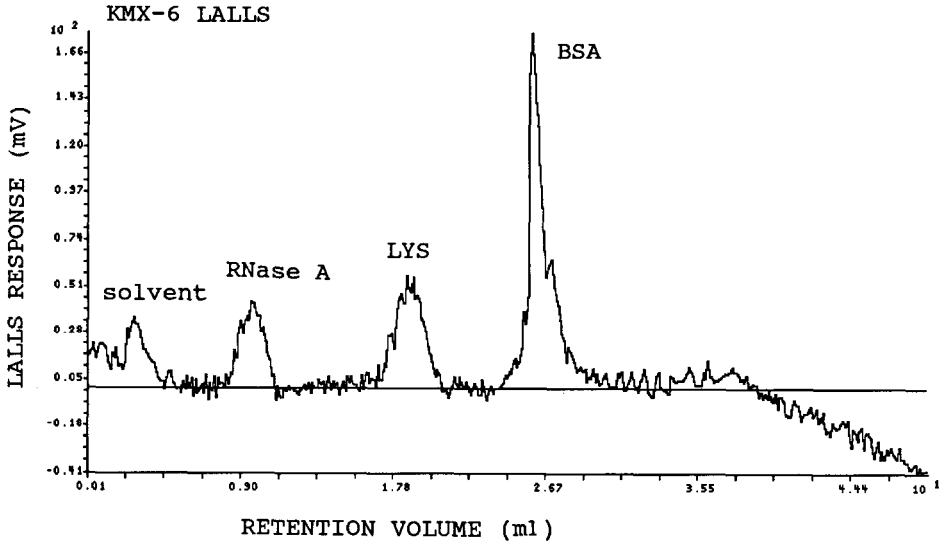
RP-HPLC–LALLS–UV chromatograms and $\bar{M}_w$ for RNase A, LYS, and BSA, eluting under various gradient times (5–45 min)

It was clear that fast gradient RP-HPLC is a practical approach to separate and isolate proteins, but does chromatography alter the biopolymer, or does it remain intact as originally assumed? There have been no discussions in the literature of what effect fast gradient RP conditions might have on the nature of the eluting species, or of what changes might incur when varying gradient formation times. It has generally been assumed that the formation of the gradient and overall time of elution do not affect the nature of the protein species eluted. However, in the absence of $\bar{M}_w$ information, these are indeed, assumed. These studies were designed to determine if LALLS photometry was compatible with on-line gradient RP-HPLC and could the approach be used to indicate/characterize the nature of the proteins eluting as a function of gradient formation time?

First attempts were to couple RP-HPLC with LALLS detection for proteins using a 20-min gradient, Fig. 1. The LALLS–UV signals were obtained free of any baseline anomalies, and background/baseline subtraction or correction procedures were not necessary. Each chromatographic condition was reproduced five times, except where noted, under identical HPLC–LALLS–UV conditions over a long period of time (days to weeks). On processing this data, Table I, it was apparent that the molecular weights obtained were different than those initially expected.

Table I contains the RP-HPLC–LALLS–UV data for these three proteins under identical chromatographic conditions while varying only gradient formation time, with the molecular weights representative of five separate injections under each condition. For gradients operating at 15 min or greater, the first two eluted proteins. RNase A and LYS, agree with literature, with %R.S.D. values often less than 10%. BSA is anomalous, since it shows a much higher $\bar{M}_w$, suggestive of a mixture of species. Careful examination of the LALLS signal of Fig. 1 indicates a shoulder on the BSA tail, which is not as apparent in the UV signal. This is because LALLS responds to the multiplicative factor of concentration and molecular weight. Low concentrations of higher order aggregates are detected more readily with LALLS since it is sensitive to both concentration and $\bar{M}_w$. Bulk concentration detection such as UV, responds to chromophore additivity in addition to concentration. RNase A and LYS, showed no evidence of chromatographic higher order species. At this point in time we were lead to believe that BSA, as received from the vendor, already contained an aggregate, most likely the dimer, perhaps even higher order species. Our studies with this particular lot of BSA, including FIA–LALLS and SEC–LALLS, strongly supported our assumptions and that this particular lot of standard BSA was a mixture of at least monomer and dimer. It is also well known that BSA aggregates readily, and that these results were not uncommon.

At gradient formation times greater than or equal to 15 min, the calculated $\bar{M}_w$ values for all three proteins remained fairly constant and within experimental error when referenced to literature. However, at both 10 and 5 min, there was a marked



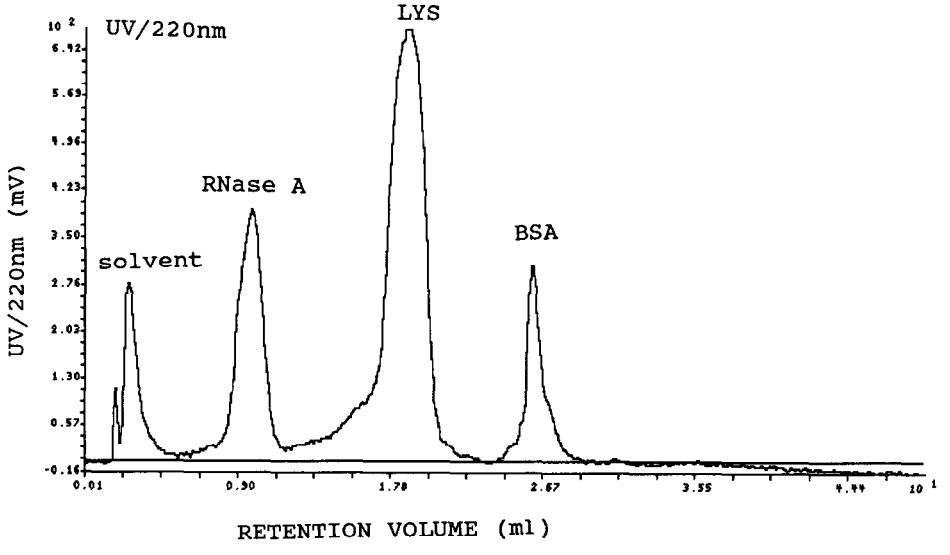


Fig. 1. RP-HPLC–LALLS–UV chromatogram of RNase A, LYS, and BSA, using a conventional 20-min, 20–55% B in A, gradient. HPLC conditions: mobile phase A = 0.15% trifluoroacetic acid, pH 3.0 and B = 0.15% trifluoroacetic acid in 95% acetonitrile–water, pH 3.0; flow-rate = 1.63 ml/min; detectors: UV = 220 nm (1000 mV f.s. = 2 a.u.f.s.) and LALLS = SEC cell, 6–7° annulus, 0.2-mm field stop, G_θ = 320 mV at initial gradient conditions, G_0 = 400 mV with $D = 4.40 \cdot 10^{-9}$; column: 1.5-μm C_8 bonded silica, non-porous, 3.6 cm × 8 mm; order of elution and injected mass = 20 μl of RNase (11.93 mg/ml), LYS (14.73 mg/ml), and BSA (3.93 mg/ml); gradient: 20–55% B in A, linear, in 20 min. Top = LALLS; bottom = UV.

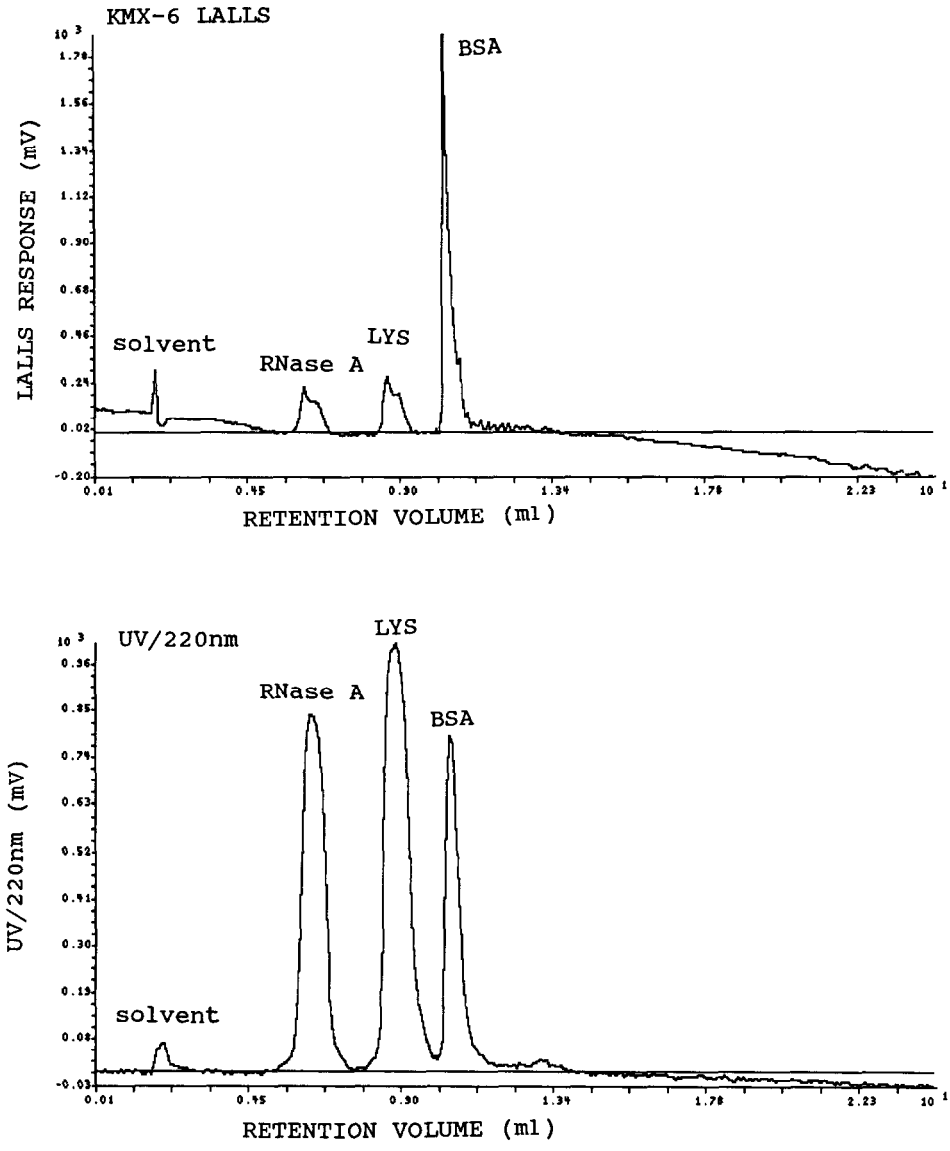


Fig. 2. RP-HPLC–LALLS–UV chromatogram of RNase A, LYS, and BSA, using a fast 5-min, 20–55% B in A gradient. Same conditions as in Fig. 1 but gradient time is now 5 min. Top = LALLS; bottom = UV.

increase in $\bar{M}_w$ for all three proteins, especially that for BSA. $\bar{M}_w$ values were derived using off-line experimentally determined refractive index, n, and specific refractive index increment, dn/dc, for each protein using as the solvent, the mobile phase composition of elution observed for each gradient formation time.

Fig. 2 illustrates typical RP-HPLC–LALLS–UV chromatograms for the three proteins using a gradient formation time of 5 min. The LALLS signal showed increased "apparent" peak splitting for all proteins not evidenced by UV. This

TABLE II

SUMMARY OF 10-MIN GRADIENT RP-HPLC LALLS–UV STUDIES FOR $\bar{M}_w$ DETERMINATION

Temperature effect on biopolymer/aggregate formation. All $\bar{M}_w$, n, and dn/dc determinations were at 633 nm.

Temperature	*Molecular weight ± S.D. (n) (dn/dc)*		
	RNase A	*LYS*	*BSA*
Ambient (25°C)	18 400 ± 1600 (1.335) (0.172)	19 800 ± 1000 (1.335) (0.182)	152 000 ± 16 400 (1.336) (0.172)
Ice–water (4°C)	19 300 ± 300 (1.339) (0.145)	11 500 ± 600 (1.340) (0.148)	156 900 ± 3700 (1.341) (0.139)
Ice–methanol (−5°C)	38 100 ± 300 (1.340) (0.133)	12 600 ± 600 (1.342) (0.133)	223 800 ± 5300 (1.344) (0.119)

phenomenon was initially thought to be due to unaccounted changes in the LALLS scattering volume giving rise to an obviously invalid LALLS response. However, after manipulating the procedure for proper cell alignment throughout the gradient, peak splitting was determined to be a function of the analyte and the chromatographic production of higher order species due to the speed of gradient formation. All data were reproducible over time to further support our findings.

These data strongly suggested that protein aggregation resulted as a function of the gradient under fast-RP conditions.

n And dn/dc values for proteins using the mobile phase composition causing elution under RP-HPLC–LALLS–UV conditions

Various RP-HPLC mobile phase combinations (%B:%A) were prepared volumetrically, and their refractive indices at 633 nm and at the required temperature were determined. Though not illustrated here, the refractive index at 25°C changed somewhat markedly in going from 0 to about 30% B, 1.333–1.343. It remained fairly linear from 30–80% B in A, 1.343–1.347. These determinations are presented, in part, in Tables II and III. This was reassuring, for it suggested that non-linear changes in n could not be responsible for any observed changes in $\bar{M}_w$.

TABLE III

FIA–LALLS STUDIES FOR $\bar{M}_w$ DETERMINATION USING VARIOUS RP BUFFER COMBINATIONS

All reported numbers are $\bar{M}_w$ ± S.D. S.D. values are standard deviations of the reciprocal of the y-intercept.

Protein	$\bar{M}_w$ ± *S.D.*	A_2 *(ml mol/g²)*	*dn/dc ± S.D. (ml/g)*	*n ± S.D. (633 nm/25°C)*
RNase A[a]	12 800 ± 400	$7.50 \cdot 10^{-2}$	0.172 ± 0.001	1.335 ± 0.001
LYS[b]	14 600 ± 900	$-5.39 \cdot 10^{-2}$	0.182 ± 0.002	1.335 ± 0.001
BSA[c]	92 600 ± 5600	$7.49 \cdot 10^{-2}$	0.172 ± 0.004	1.336 ± 0.001

[a] 25% B:A buffer combination.
[b] 35% B:A buffer combination.
[c] 42% B:A buffer combination.

The dn/dc values for each protein as a function of the water–acetonitrile composition were experimentally determined in the traditional manner, and plotted, though not shown here, for a large number of individual $\Delta n/\Delta c$ *vs.* c points. Tables II and III include summarized dn/dc data, and these values were used to calculate individual protein $\bar{M}_w$ values. It was again reassuring that the individual dn/dc values in various %B:A RP buffer combinations were quite constant for RNase A, LYS, and BSA.

The two most suggestive reasons for the observation of the increasing $\bar{M}_w$ for these proteins, as a function of the speed of the gradient formation, namely unaccounted for changes in n and dn/dc, appeared not to be involved. In eliminating other plausible interpretations, it appeared that the increases in $\bar{M}_w$ were real, and due to biopolymer aggregate formation. However, it remained to unequivocally demonstrate that this was true through additional studies. Several major studies will be described: (1) orthogonal chromatography of protein peaks eluted under 5-min gradients, into a 20-min gradient experimentally determined not to cause aggregate formation; (2) low temperature $\bar{M}_w$ determinations under 10-min gradient conditions, and comparing the above determined sets of $\bar{M}_w$ data for these proteins; (3) concentration related $\bar{M}_w$ determinations under 10-min gradient conditions; (4) SEC–LALLS–UV–DRI determinations of $\bar{M}_w$ using modified protein buffer conditions known to theoretically prevent aggregation and association; and (5) FIA–LALLS determinations of $\bar{M}_w$ and A_2 under various isocratic water–acetonitrile mobile phase conditions.

Orthogonal RP-HPLC–UV–RP-HPLC–LALLS–UV chromatography for individual $\bar{M}_w$ determinations

It was possible that the increased $\bar{M}_w$ observed at fast RP gradient elution times (5–10 min) were yet due to an artifact of the analytical approach. In order to further determine if real aggregates were formed, two additional chromatographic studies were undertaken. In the first, orthogonal chromatography was used for each individual protein eluting under a 5-min gradient, and then this protein was directly valve switched into the start of a 20-min gradient (Fig. 3, Experimental). A UV detector was placed after the first 5-min RP column to indicate protein elution. This protein was then valve switched into the 20-min gradient, at the end of which was the usual series of LALLS–UV detection. Only two plausible outcomes for this study were possible. The protein aggregate, if indeed present and kinetically stable at the initial 5-min chromatographic time scale, *would* or *would not* survive the second 20-min gradient run and elute as the previously observed $\bar{M}_w$ from the direct 5- or 20-min run. It was also possible that, protein dependent, some of the proteins would exhibit one form, or another, of aggregation behavior.

Table IV summarizes the results of this study represented as $\bar{M}_w \pm$S.D. in g/mol or daltons. In the case of RNase A, $\bar{M}_w$ was determined to be 24 700(1700), which was slightly above the 21 700 observed from the direct 5-min run, and substantially above a direct 20-min gradient (14 500). For LYS, the $\bar{M}_w$ was 24 500 (2400), almost identical to the value found for the direct 5-min run alone (25 600). This was again above the value for LYS in a direct 20-min run (13 800). Thus, the first two proteins exhibited behavior under the orthogonal gradient conditions coinciding with the presence of aggregates formed in the 5-min gradient. Therefore, the 5-min gradient formed

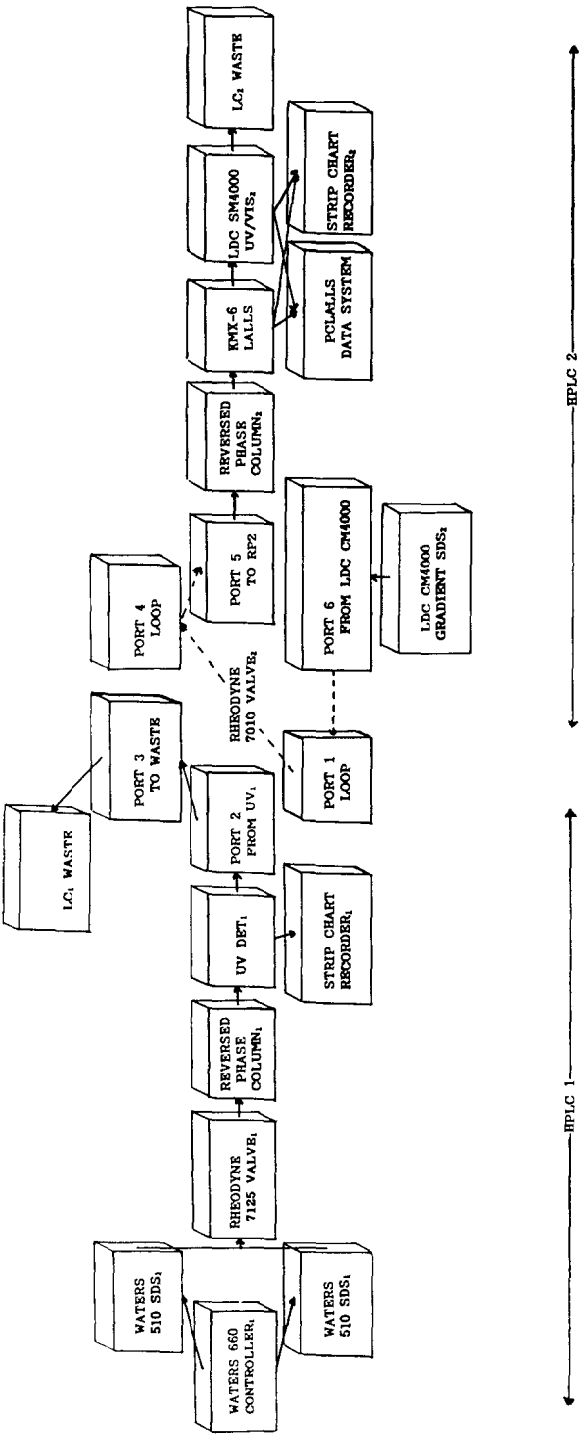


Fig. 3. Schematic block diagram of orthogonal RP-HPLC–UV–RP-HPLC–LALLS–UV instrumental system.

TABLE IV

MOLECULAR WEIGHT DETERMINATIONS FOR ORTHOGONAL CHROMATOGRAPHIC STUDIES WITH RNase A, LYS, AND BSA, AS COMPARED TO OTHER STUDIED RP CHROMATOGRAPHIES

Orthogonal conditions: RP 1: 5-min gradient into RP 2: 20-min identical gradient. All reported numbers are $\bar{M}_w \pm$ S.D.

Protein	*Orthogonal* $\bar{M}_w \pm$ *S.D.*	*Fast 5 min* $\bar{M}_w \pm$ *S.D.*	*Conv. 20 min* $\bar{M}_w \pm$ *S.D.*
RNase A	24 700 ± 1700	21 700 ± 700	14 500 ± 1300
LYS	24 500 ± 2400	25 600 ± 1100	13 800 ± 1300
BSA	85 400 ± 2100	249 000 ± 12 600	93 500 ± 3900

protein aggregate remained essentially intact throughout the second 20-min gradient chromatographic timescale.

In the case of BSA, its $\bar{M}_w$ after the orthogonal analysis was 85 400 (2100) daltons, substantially lower than the value of 249 600 daltons observed in the direct 5-min run alone. This orthogonal chromatography determined value was almost identical, within experimental error, to the value for BSA observed on a direct 20-min run. BSA behaved differently when its aggregates had formed in the 5-min gradient. These species may not have been of sufficient lifetime or stability to elute intact from the 20-min gradient. It seems reasonable to assume that aggregate stability under a 20-min gradient run would be species/protein dependent. The fact that RNase A and LYS behaved similarly, as opposed to BSAs behavior, is again reasonable given the extreme differences in $\bar{M}_w$ values, primary structures, as well as higher order structures.

The above results were all consistent of the assumption that aggregates of at least RNase A and LYS were formed in the 5-min, and most likely the 10-min, RP gradients. It is probable that BSA had also formed aggregates under the 5-min gradient but its orthogonal studies did not support this assumption. It seems unlikely that the observations for BSA were due to experimental artifacts of this approach, but rather had much more to do with the kinetics of aggregate formation and dissociation under the chromatographic conditions utilized.

Fig. 4 illustrates the type of chromatograms obtained for the above orthogonal gradient runs, using RNase A as the illustrative protein. With respect to the LALLS signal, there is evidence of a lower retention volume shoulder co-eluting with the principal peak. This is not as apparent when the UV signal is viewed. Similar observations were made for BSA, suggestive of the presence of a mixture of monomer and dimer, since its $\bar{M}_w$ was always higher than that of monomer alone. This particular BSA sample never yielded a correct $\bar{M}_w$, under any HPLC or FIA–LALLS conditions, suggestive of pure monomer.

Low-temperature studies in RP-HPLC–LALLS–UV for RNase A, LYS and BSA

Determination of $\bar{M}_w$ *under fast gradient RP elution conditions (10 min).* All of the above studies were consistent with the presence of aggregates formed under fast (5–10 min) gradient RP elution chromatography. Another study was suggested[52] that involved determining $\bar{M}_w$ at lower temperatures, using a gradient formation time

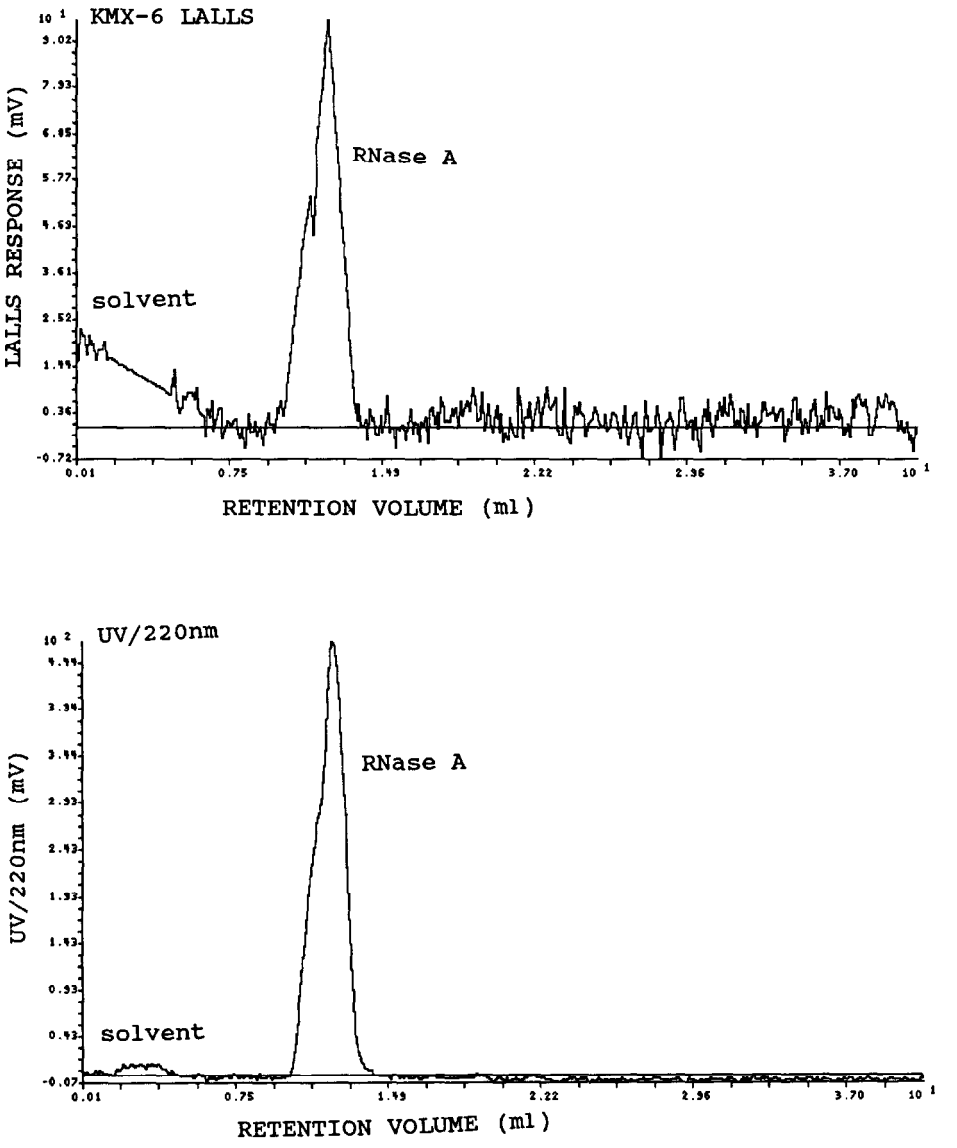


Fig. 4. Orthogonal RP-HPLC–UV–RP-HPLC–LALLS–UV chromatogram for RNase A, first injected into a 5-min gradient from 20–55% B in A, then valve switched into a 20-min gradient from 20–55% B in A. First HPLC conditions: flow-rate = 1.63 ml/min; detector: UV = 220 nm (1000 mV f.s. = 2 a.u.f.s.); column: 1.5-μm C_8 bonded silica, non-porous, 3.6 cm × 8 mm I.D.; order of elution and injected mass = 20 μl of RNase A (10.32 mg/ml), LYS (10.52 mg/ml), and BSA (4.05 mg/ml); gradient: 20–55% B in A, linear, in 5 min. Second HPLC conditions: Same operating conditions as first, except gradient: 20–55% B in A, linear, in 20 min and the addition of LALLS detection post column/pre-UV using the SEC cell, 6–7° annulus, 0.2-mm field stop, G_θ = 320 mV at initial gradient conditions, G_0 = 400 mV with $D = 4.40 \cdot 10^{-9}$. LALLS solvent front peak removed in order to better illustrate the orthogonal RNase A peak. Top = LALLS, bottom = UV.

suggesting the formation of aggregates (*i.e.*, 10 min). If these were really aggregates, and not artifacts of the system/approach, then similar analyses at lower temperatures should form even higher aggregated species, at least theoretically. As protein solubility decreases with lower temperatures, their propensity to aggregate increases accordingly. For most proteins, this is a well known phenomenon. We have performed two low-temperature studies with Table II summarizing the $\bar{M}_w$, n, and dn/dc values for all three proteins in a 10-min gradient run at ice–water temperatures (*ca.* +4°C) and at ice–methanol temperatures (*ca.* −5°C).

At ice–water conditions (4°C) both RNase A and BSA remained unchanged in their $\bar{M}_w$ values over ambient temperature, however, LYS decreased. At ice–methanol (−5°C) temperatures, both RNase A and BSA showed much higher $\bar{M}_w$ values in comparison with both the room temperature and ice–water runs. LYS remained unchanged with respect to ice–water conditions, an apparent anomaly. The LYS peak produced a higher retention volume shoulder, as evidenced from the UV signal, but due to the lack of ample LALLS signal, this shoulder can be assumed to be a much lower $\bar{M}_w$ impurity present in the standard sample. Since LYS is highly protonated at pH 3.0 (its pI = 11.0), and colder temperatures generally promote protein aggregation, one must also consider that LYS could have been partially adsorbed to the column support. This would result in under-estimating $\bar{M}_w$ since eqn. 5 states that $\bar{M}_w$ is inversely proportional to concentration. After inspection of the chromatogram, Fig. 5, it became apparent that both LALLS and UV peak shapes for RNase A were atypical. What were single UV and split LALLS peaks for RNase A, were now more pronounced and easily identifiable as multiple species peaks.

LALLS peak shapes for BSA, as evidenced by UV, were well behaved at both sub-ambient temperatures, though additional peaks appeared at −5°C for RNase A, by both LALLS and UV. It was clear that the first eluting protein, RNase A, had been split into at least three separate species, and BSA (last eluter) had a tailing shoulder, as always. The LALLS signal for LYS at −5°C in a 10-min gradient run had become quite broadened, very different from what was always observed for this protein at higher temperatures. This was also apparent for RNase A from the UV signal. We have seen that LYS apparently behaved quite differently under very low temperature fast gradient conditions, not aggregating like the others.

One other point of information for these low temperature studies was that the retention times for all three proteins had shifted by about +3% B in comparison with ambient temperature. This suggested the increased formation of aggregates (increased number of hydrophobic patches on the surface) under colder column conditions. All of the low-temperature studies were consistent, and that most aggregates increased proportionally at the lower temperatures.

Concentration studies in RP-HPLC–LALLS–UV for RNase A, LYS and BSA

Determination of $\bar{M}_w$ under fast gradient RP elution conditions (10 min). To further support our experimental findings, one last reversed-phase study was undertaken, where all conditions were held constant using fast gradient 10-min elution conditions, while varying biopolymer injected mass. The injected masses were in the range of 40–300 μg depending on which biopolymers were chromatographed (Table V). Accordingly, all retention volumes increased slightly (≈1% B for RNase A and LYS, while ≈2% B for BSA) in addition to their $\bar{M}_w$ values with increasing injected

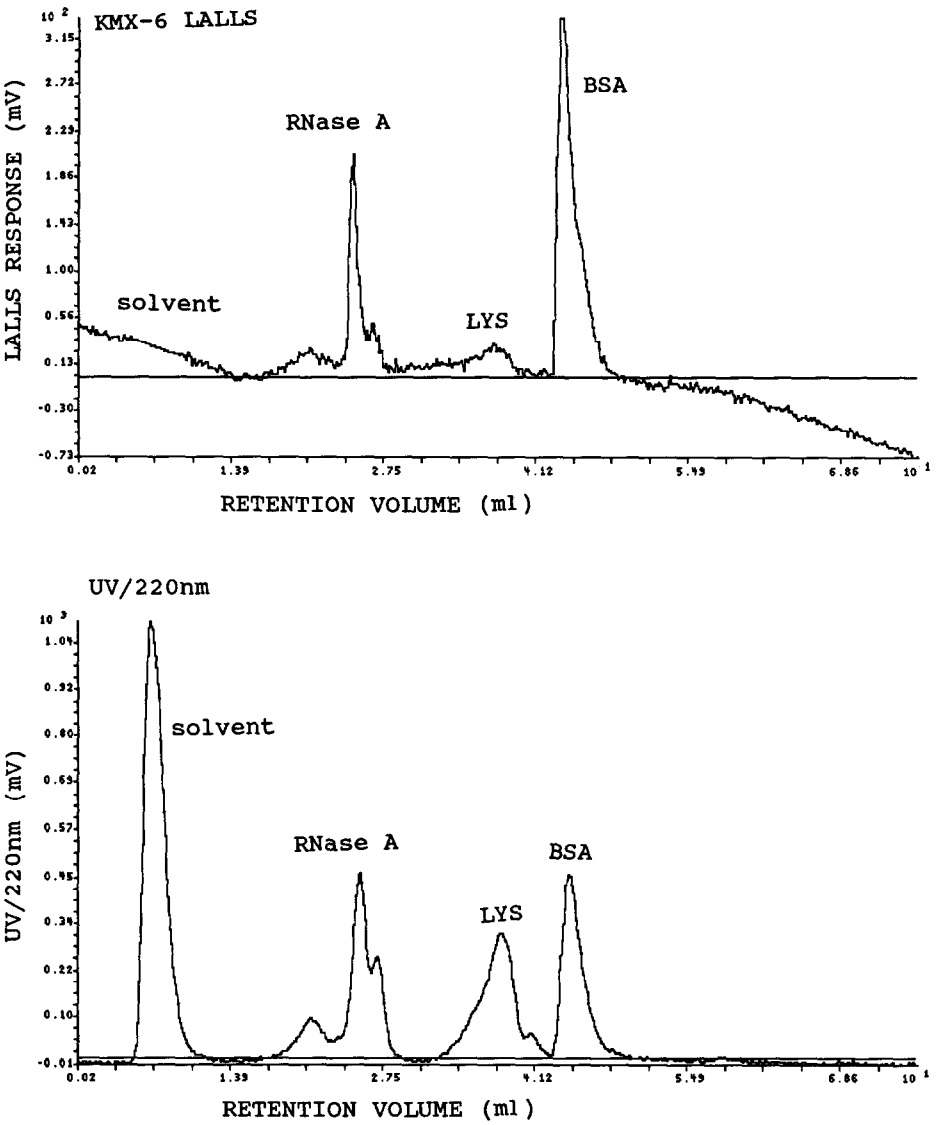


Fig. 5. Cold RP-HPLC–LALLS–UV chromatogram of RNase A, LYS, and BSA, using a 10-min, 20–55% B in A, gradient. Same conditions as in Fig. 1, but temperature is now −5°C. Top = LALLS, bottom = UV.

mass. Though not illustrated here, when $\bar{M}_w$ was plotted *versus* injected mass, and linear regression was performed, the corresponding *y*-intercepts (a condition of infinite dilution) yielded $\bar{M}_w$ values close to that of its monomer. These data further confirm our findings that under certain fast RP-HPLC conditions, proteins have a tendency to aggregate.

TABLE V

SUMMARY OF 10-MIN GRADIENT RP-HPLC–LALLS–UV STUDIES FOR $\bar{M}_w$ DETERMINATION

Concentration effect on biopolymer/aggregate formation.

Injected mass (μg)	*Molecular weight ± S.D. (5 determinations)*		
	RNase A	*LYS*	*BSA*
40	—	—	120 000 ± 4800
60	—	—	135 000 ± 5000
80	—	—	147 000 ± 6100
100	15 800 ± 900	16 000 ± 800	168 000 ± 4900
120	—	—	180 000 ± 6200
150	16 700 ± 1000	16 700 ± 1000	—
200	17 800 ± 1200	17 500 ± 1000	—
250	18 700 ± 700	18 600 ± 900	—
300	20 000 ± 800	19 500 ± 900	—

FIA–LALLS studies in RP-HPLC–LALLS buffers at ambient temperature

In order to better understand the intact nature of the proteins present, as supplied by the vendor, FIA–LALLS studies were performed in various %B:A RP buffer combinations as determined in the previous RP-HPLC–LALLS–UV experiments. Plots of the scattering function $Kc/\bar{R}_\theta$ *versus* concentration were constructed for each protein, as illustrated in Fig. 6. Each protein was prepared in a mixture of %B:A equal to that which resulted in elution under gradient RP conditions. Thus, Table III summarizes all of the $\bar{M}_w$ data obtained for each individual protein, as well as its

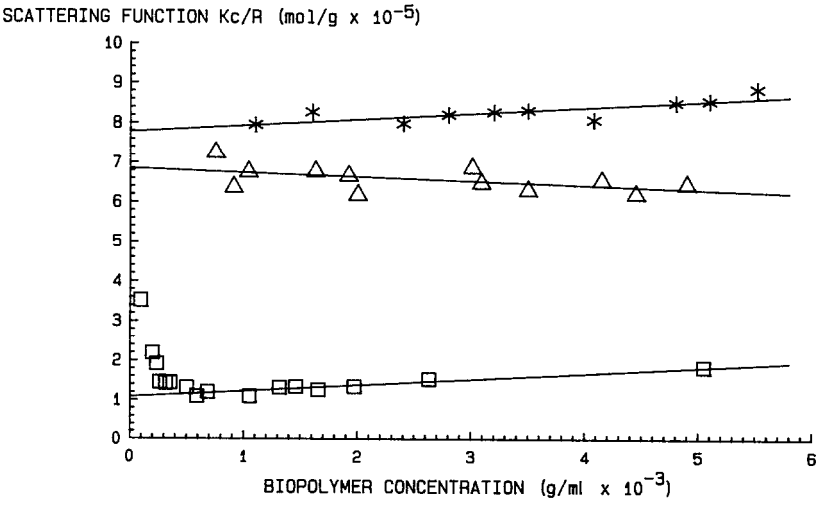


Fig. 6. FIA–LALLS (via Chromatix KMX-6) plots for RNase A, LYS, and BSA. LALLS conditions: 6–7° annulus; 0.2-mm field stop; G_θ = 115 mV; G_0 = 100 mV with $D = 3.67 \cdot 10^{-9}$; flow-rate = 0.1 ml/min (nominal); solvent/carrier = 25% B in A (RNase A), 35% B in A (LYS), and 42% B in A (BSA). * = RNase A ($\bar{M}_w$ = 12 800 ± 400, $A_2 = 7.5 \cdot 10^{-2}$); △ = LYS ($\bar{M}_w$ = 14 600 ± 900, $A_2 = -5.39 \cdot 10^{-2}$); □ = BSA ($\bar{M}_w$ = 92 600 ± 5600, $A_2 = 7.49 \cdot 10^{-2}$).

second virial coefficient, A_2. RNase A exhibited a $\bar{M}_w$ of 12 800 (400) daltons, similar to the accepted literature value of 13 700, BSA's $\bar{M}_w$ was 92 600 (5600), while LYS showed a value of 14 600 (900) daltons.

It should be noted that LYS was, experimentally, the most difficult to analyze via the FIA–LALLS technique, prone to indeterminate error. This could be due to the fact that LYS is in the highly protonated form at pH 3.0. The resultant linear regression analysis was the product of numerous sample concentrations that were statistically treated using the t test with a 95% confidence interval.

Others have described deviations in $\bar{M}_w$values for numerous proteins as a function of inappropriate buffers[42,44,45]. The A_2 values for RNase A and BSA were positive, suggesting good solubility (good protein–buffer interactions). LYS, on the other hand, showed an A_2 value that was negative, but of the same order of magnitude, suggesting poor protein–buffer interaction and more protein–protein interaction. We and others have demonstrated that negative A_2 values are often suggestive of non-ideal protein–buffer interaction.

This particular lot of BSA had never shown itself to be pure monomer, as supplied by the vendor. It has consistently exhibited the contributions of higher order aggregates. As supplied, BSA existed as both a mixture of about 70% monomer and 30% dimer (FIA–LALLS $\bar{M}_w$ = 92 600 daltons), though higher aggregates may have been present. It behaved anomalously, at lower concentrations, as evidenced by plotting the data. Its scattering function $Kc/\bar{R}_\theta$ (inverse of $\bar{M}_w$) increased exponentially as concentration was lowered below 0.30 mg/ml. This can be interpreted: (1) as concentration decreases, the fraction of BSA containing the dimer dissociates into monomer, or (2) more evidently, as concentration goes below 0.30 mg/ml, errors associated from indeterminate sources have higher probability. In other words, for this particular biopolymer–buffer system studied, we would have exceeded the linear detection limit in FIA–LALLS of approximately 0.30 mg/ml. We therefore extrapolated the data above this concentration, in order to deduce $\bar{M}_w$ in the customary manner.

SEC–LALLS at ambient temperature using a disaggregating buffer system for the determination of protein $\bar{M}_w$

In order to more fully define the exact nature of the protein species present on receipt, and to support the above results on the FIA–LALLS studies, we performed conventional SEC–LALLS–UV–DRI determinations for $\bar{M}_w$ in a special buffer. This particular buffer has been described and used to prevent aggregate formation and dissociation[9,51,53]. Under these particular SEC conditions, using this buffer as the mobile phase, $\bar{M}_w$ values were determined for each protein. Work with LYS under these conditions, at pH 7.0 for the disaggregating buffer, showed adsorption. The basic protein LYS has been shown to be a sensitive probe for the determination of residual silanols represented as SiO^-, as shown by Pfannkoch *et al.*[54]. In buffers whose pH is less than or equal to 7, LYS has a strong net positive charge involving hydrophobic and electrostatic attractive forces between the protein and substrate making it an ideal probe in studying residual SiO^-, but not particularly well suited for true SEC separations utilizing silica stationary phases. This phenomenon is chromatographically undesirable and subsequent $\bar{M}_w$ determination was not possible for LYS under these chromatographic conditions. However, much of the literature on the SEC of

proteins involves derivatized silica packings, and the TSK SW series show the most popularity[55–57], even though LYS was retained.

Assuming a 90% purity for RNase A (claimed by the vendor), its $\bar{M}_w$ was exactly as that in the literature, 13 800(300) *vs.* 13 700 daltons, respectively. In the case of BSA, once again a purity assumption of 95%, claimed by the vendor, yielded a $\bar{M}_w$ of 86 900(2200) daltons, significantly above that reported for the monomer, 65 000. This had always been the case for our BSA. It had always existed as a mixture of BSA-mers. We have now been able to unequivocally characterize each peak present under these SEC–LALLS–UV–DRI conditions, and it is inclusive that this particular lot of BSA consisted of approximately 70% monomer and 30% dimer, having $\bar{M}_w$ values of 67 000(1700) and 138 000(3500) daltons, respectively, with % RSD values of less than 3%.

CONCLUSIONS

From this study, certain interesting observations have been made. After having ruled out factors such as incorrect measurements of refractive index and dn/dc leading to artifacts of the system which would lead to apparent molecular weights, one may conclude that the higher molecular weights are due to on-column aggregation and are protein specific. Though it is not fully understood at this point as to why aggregates would form as a function of the rapidity of the gradient, a certain hypothesis can be made to explain this phenomenon.

Proteins that have been studied in these experiments are hydrophilic. In the presence of hydrophobic solvents the protein molecules have a tendency to attract one another, to a point where precipitation may occur. In case of the 5- and 10-min gradients the percentage of the organic modifier changes very rapidly at the rate of about 5–7% B per min. The sudden increase in the hydrophobicity of the environment could be the reason for the protein molecules to aggregate. Apart from this, it is very difficult to imagine, on a molecular level, the behavior of proteins in a rapidly changing, dynamic environment.

The purpose of this study was to determine if it was possible to couple RP-HPLC with LALLS for doing on-line separation and characterization of proteins in a short period of time. The results obtained with shallower gradients, 15-min (less than a change of 2% B/min), do indicate that it is possible to separate and characterize proteins in a reasonable amount of time. However, using shorter gradients to speed up the analysis has serious drawbacks. In doing future work on separating proteins, optimization of the gradient range and time would be of major consideration. Though this study has established the fact that gradient RP-HPLC is compatible with LALLS under optimum conditions, further work needs to be done to investigate the precise reasons behind the formation of higher order species as a function of the rapidity of the gradient. This would involve some kind of molecular modeling to investigate the changes in the structures of the molecules under certain experimental conditions.

SYMBOLS AND ABBREVIATIONS

A_2	= second virial coefficient in units of ml mole/g^2
a.u.f.s.	= absorbance units full scale
BSA	= bovine serum albumin

c = concentration, usually expressed in mg/ml, or g/ml $\times 10^{-3}$
CMX-10A = "Chromatix Products" 12 bit analog-to-digital converter
D = transmittance of optical attenuators used in measuring the incident illuminating light beam
dn/dc = specific refractive index increment, expressed in ml/g
DRI = differential refractive index, a concentration detection method
ED = electrochemical detection
FIA–LALLS = flow injection analysis coupled to low-angle laser light scattering photometry for determination of $\bar{M}_w$ and A_2 of bulk solutions (a modern derivation of the traditional static LALLS experiment)
FL = fluorescence, concentration sensitive detection method
G_θ = intensity of light scattered from the sample at an angle, θ
G_0 = intensity of laser light beam transmitted through the sample at zero angle
HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIC = hydrophobic interaction chromatography
HPLC = high-performance liquid chromatography
K = the polymer optical constant and for measurements below 7° = $(4.079 \cdot 10^{-6})\ (n)^2(dn/dc)^2$ in units of mol cm^2/g^2
KMX-6 = LDC Analytical/Thermo Instruments, "Chromatix Products" LALLS photometer
KMX-16 = LDC Analytical/Thermo Instruments, "Chromatix Products" instrument used to physically measure dn/dc
LALLS = Low-Angle Laser Light Scattering
LDA = linear diode array, a concentration sensitive detection method (UV)
LYS = lysozyme
-mers = monomer repeat units
MS = mass spectrometry
mV f.s. = millivolts full scale
$\bar{M}_w$ = weight-average molecular weight in units of g/mole or daltons
n = solvent/mobile phase refractive index, determined at 633 nm
PCLALLS and MOLWT3 are software packages available through LDC Division/Thermo Instruments, and are used to process on-line LALLS measurements
RNase A = ribonuclease A
RP-HPLC = reversed-phase high-performance liquid chromatography
R.S.D. = relative standard deviation = S.D./mean, sometimes expressed as %R.S.D.
R_θ = Rayleigh factor
$\bar{R}_\theta$ = excess Rayleigh factor = Rayleigh factor of the sample solution − Rayleigh factor for solvent alone
S.D. = standard deviation using $n-1$ sampling
SEC = size exclusion chromatography
SEC–LALLS–UV–DRI = SEC coupled to LALLS, UV and DRI detection, in series
$(\sigma' l')^{-1}$ = solid angle, scattering volume correction, defined by instrument geometry and solvent refractive index
UV–VIS = ultraviolet–visible, a concentration detection method

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