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# Application of low-angle laser light scattering detection in the field of biochemistry

## Review of recent progress

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SUMMARY

Among various detectors now available, the low-angle laser light scattering photometer has not attracted much attention. This unique detector, however, affords invaluable information on the characterization of biopolymers with respect to their molar mass.

## INTRODUCTION

Molar masses of proteins have traditionally been determined by physico-chemical techniques, such as osmometry, ultracentrifugation and light scattering. The introduction of techniques that depend on the molecular sieve effect of gels has changed the situation. Nowadays, biochemists chiefly depend on techniques such as gel permeation chromatography (GPC) and gel electrophoresis for the molar mass determination of proteins and nucleic acids. However, the size of a macromolecule is not always a unique function of its molar mass and the realization of this situation has led to reconsideration of molar mass determination through an approach with a secure physico-chemical basis.

About 10 years ago, we had the strong feeling that a convenient and reliable technique for molar mass determination was necessary other than the classical physico-chemical techniques. We became interested in the low-angle laser light scattering (LALLS) technique, developed in 1971 by Kaye *et al.*[1], as a promising candidate. When used as a detector for GPC, it was regarded as the most accurate approach for the determination of the molar mass distribution of polymers. The impact of the new technique was limited, however, and it failed to be accepted in the field of biopolymer research, owing to the absence in those days of GPC columns suitable for aqueous solvents and to the ignorance of using aqueous solvents in the construction of the initial models of commercial instruments.

We began studies on the introduction of the LALLS technique into the field of biochemistry and cooperated with Tosoh in an effort to develop a LALLS photometer

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suitable for the purpose, helped considerably by the simultaneous development of GPC columns for aqueous solvents by that company. This paper reviews those efforts but avoiding overlap with a previous review[2].

## TYPICAL EXPERIMENTAL PROCEDURE

A Model LS-8000 low-angle laser light scattering photometer (LALLS detector) from Tosoh is used in the author's laboratory. It is compact (620 × 310 × 330 mm) and weights 37 kg. The light source is a 5-mW helium–neon laser with a wavelength of 633 nm. The flow cell with an internal volume of 30 $\mu$l (10 mm × 2 mm I.D.) can be thermostated by a constant-temperature water-jacket. Forward scattering from a 0.1-$\mu$l volume is collected at an angle of *ca.* 5°. With respect to sensitivity, 50 $\mu$g of bovine serum albumin with a molar mass of 66 300 gave a sufficient pen deflection. The amount of bovine serum albumin is one tenth of that required in the initial model (LS-8). The LALLS detector can accommodate a precision differential refractive index (RI) detector (Model RI-8011: light source, photodiode with an effective wavelength of 660 nm) in a single body.

Fig. 1 shows the outline of the optics of the Model LS-8000 LALLS detector. The baseline stability was significantly improved by the shift of the monitoring point of the light source from the rear side of the cell traditionally positioned there[1] to the front of the cell.

Fig. 2 shows the most refined system we are now using. The degasser (DG) is provided with a long PTFE tube surrounded by a reduced-pressure atmosphere, thus efficiently eliminating air dissolved in the solvent. Although recent models of pumping devices produce a pulseless flow, in our experience installation of a damping system consisting of a damper (Da) and a tube with a narrow orifice after the pump is necessary for extension of the life time of the usually expensive column. The columns (GC and MC), flow cell of the LALLS detector and the tubing connecting them are thermostated. The tubing was placed between a pair of plastic tubes with walls of high

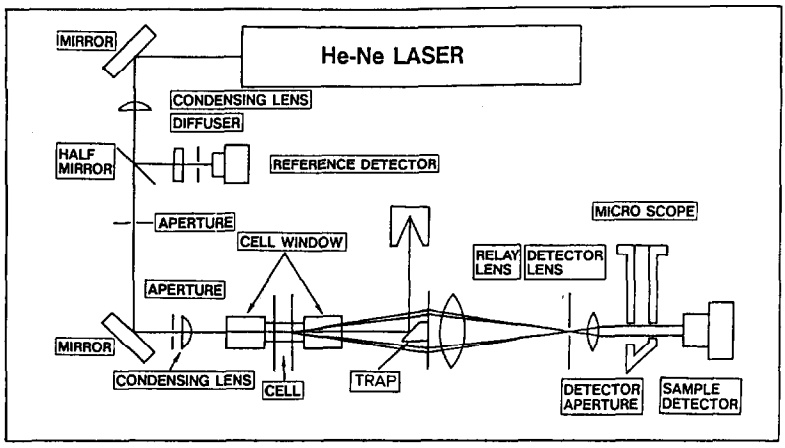


Fig. 1. Optics of the TSK Model 8000 LALLS detector.

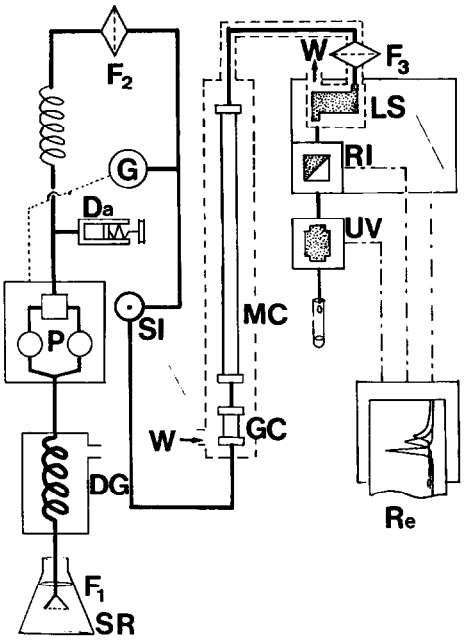


Fig. 2. Schematic diagram of the instrumentation. SR = solvent reservoir; $F_1$ = sintered stainless-steel filter (Umetai Seiki, Model SFY); DG = degasser (Erma Optical Works; Model ERC-3310); P = dual pump (Tosoh, CCPD); Da = bellows-type damper (Umetani Seiki, Model S-100) with a helically coiled stainless-steel tube (2 m × 0.1 mm I.D.) on the downstream side; G = pressure gauge (Umetani Seiki) provided with a safety device to shut down the pump when the pressure exceeds the limit value for the column; $F_2$ = sintered stainless-steel filter (Umetani Seiki, Model SLF); SI = sample injecter with a sample loop of 100-μl internal volume; GC = guard column (Tosoh, TSK-GEL GSWXL, 4.0 cm × 6.0 mm I.D.); MC = main column (Tosoh, TSK-GEL G3000SWXL, 30 cm × 7.8 mm I.D.); $F_3$ = ultrafilter with pore size of 0.5 μm (Millipore, type FHLP 01300); LS = LALLS detector (Tosoh, LS-8000); RI = RI detector (Tosoh, RI-8011); UV = UV detector (Tosoh, UV-8000); Re = three-pen recorder. The region enclosed by the dashed lines is thermostated by circulation of constant-temperature water.

heat conductivity and semicircular cross-section. The provision for thermostating is essential when a sample undergoes temperature-dependent association–dissociation.

Compared with the RI and UV detectors, each with a cell of internal volume 10 μl, the LALLS detector (LS) has cell volume of 30 μl, and hence broadening of a peak during passage through this cell is more significant than that for either of the other detector cells. When thermostating is not required, the UV detector is therefore preferably installed in advance of the LALLS detector. The RI detector should not be installed before the LALLS detector, because temperature of the RI detector cell is set about 10°C higher than room temperature for strict thermostating. Installation of the RI detector before the LALLS detector results in supplying a solution or solvent of a higher temperature to the LALLS detector, which is sensitive to temperature fluctuations. Further details of the system are described in the legend to Fig. 1.

The use of the LALLS detector requires greater care than that required in ordinary high-performance liquid chromatographic (HPLC) experiments, primarily because LALLS detector gives a signal that is proportional to the product of molar

mass and weight concentration, which implies that any dust particles will give an enormous noise, and a small air bubble will also act as a particle because of the significant difference in the refractive indices of air and the solvent. Dusts and air bubbles must be therefore removed with extreme care. The higher the molar mass of the sample, the easier is the measurement using the LALLS detector. When the molar mass of a sample is an order of $10^6$, an extremely low sample concentration is required even at the lowest sensitivity setting. In such a situation, measurements must be carried out in a well thermostated room to stabilize the RI detector, which is equipped with a thermostated cell but still requires further thermostating of the whole equipment in operation at such a high sensitivity. When these prerequisites are fulfilled, measurements can be carried out routinely using a system such as that shown in Fig. 2.

## DATA HANDLING

As the result of improvements to the LALLS detector, measurements can now be carried out at a concentration low enough to make consideration of the concentration dependence unnecessary. Outputs of the three detectors, designated (LS), (RI) and (UV), respectively, are related to the molar mass of a sample according to the following equation, which is derived from the classical Rayleigh equation[3]:

$$M = k \cdot \frac{(\mathrm{LS})}{(\mathrm{d}n/\mathrm{d}c)(\mathrm{RI})} \tag{1}$$

This expression is valid when both the protein concentration and scattering angle are low enough to allow neglect of the concentration-dependent terms in the Rayleigh light scattering equation[3]. Using the system shown in Fig. 2, these requirements are satisfied in most instances.

For a sample detectable by the UV detector, the value of the specific refractive increment, $(\mathrm{d}n/\mathrm{d}c)$, can be assumed to be proportional to $(\mathrm{RI})A/(\mathrm{UV})$, where $A$ is molar absorptivity of the sample, Eqn. 1 can be accordingly modified to give the following equation:

$$M = k' \cdot \frac{(\mathrm{LS})(\mathrm{UV})}{A(\mathrm{RI})^2} \tag{2}$$

This is the general equation applicable to most proteins. Determination of the value of $A$ is a crucial prerequisite and requires great patience and skill. This is an unavoidable step in determination of molar mass when the particular protein cannot be assumed to be a simple protein, as in examples which will be described later.

When $\mathrm{d}n/\mathrm{d}c$ is assumed to be equal for samples of unknown molar mass and for standard samples of known molar mass, eqn. 1 can be simplified to give the following equation:

$$M = k'' \cdot \frac{(\mathrm{LS})}{(\mathrm{RI})} \tag{3}$$

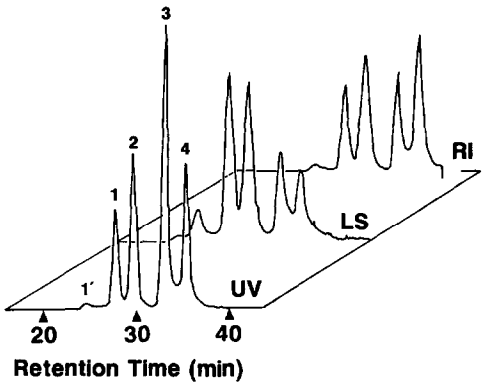


Fig. 3. Typical example of elution curves obtained with UV, LS and RI detectors. The numbers in parentheses are the amounts of the proteins applied. 1 = Bovine serum albumin (50 μg; 1', dimer); 2 = ovalbumin (60 μg); 3 = carbonic anhydrase (75 μg); 4 = ribonuclease (100 μg).

For water-soluble globular proteins that do not contain significant amounts of non-amino acid components, the above assumption is valid as a first approximation. Furthermore, eqn. 3 can even be modified to give the following equation when only a dissociation–association phenomenon of a particular protein is involved:

$$M = k''' \cdot \frac{(\mathrm{LS})}{(\mathrm{UV})} \tag{4}$$

We have carried out a series of studies in wich the most appropriate equation among eqns. 1–3 was utilized in each. An example is given shown below. Fig. 3 shows three elution curves obtained by the three detectors for four proteins. Another run was made with a mixture containing different set of four proteins. For these proteins, $dn/dc$ can be safely assumed to be identical. Fig. 4 shows plot of the (LS)/(RI) ratios for the

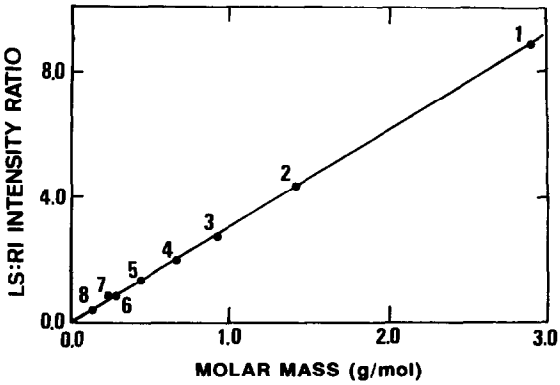


Fig. 4. Plot of the ratio of output of the LALLS detector to that of the RI detector. Proteins with molar masses in parentheses: 1 = yeast glutamate dehydrogenase (290 000); 2 = hog heart muscle actate dehydrogenase (142 000); 3 = yeast enolase (93 345); 4 = bovine serum albumin (66 300); 5 = ovalbumin (44 000); 6 = bovine carbonic anhydrase (29 000); 7 = yeast adenylate kinase (21 500); 8 = bovine pancreatic ribonuclease (13 700).

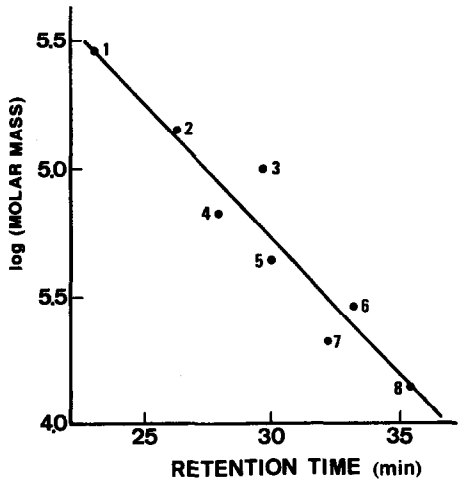


Fig. 5. Plot of logarithms of molar masses of the proteins in Fig. 4 against their retention times.

total of eight proteins against their molar mass. The linearity of the plot clearly demonstrates the efficiency of the approach in the determination of the molar mass of such proteins. Such a plot can be used as a calibration line for the determination of the molar mass of an unknown protein provided that it can be assumed to have the same value of $dn/dc$ as the standard proteins.

A plot of log (molar mass) against retention time gave scattered points as shown in Fig. 5. The line tentatively drawn through the points can hardly be used as a calibration line.

## APPLICABILITY OF GPC–LALLS IN BIOCHEMISTRY

The result described above is only an example of the application of the GPC–LALLS system. Comparison of Figs. 4 and 5 clearly shows that the GPC–LALLS technique is far superior to the conventional approach, where molar mass is determined simply from retention time.

The molar mass determination of membrane proteins is one of the most fascinating fields of application of the technique. Confirmation of the trimeric nature of porin, an *E. coli* outer membrane protein, solubilized by either sodium dodecyl sulphate (SDS)[4] or octaethylene glycol *n*-dodecyl ether[5] was the initial example of the successful application in the approach. The technique was further applied to the characterization of the nature of $Na^+$, $K^+$-ATPase solubilized by SDS[6] or the non-ionic surfactant described above[7]. The methodology of the application of GPC–LALLS to membrane proteins was reviewed recently[8].

The approach was further extended recently to monitoring the molar mass of ATPase just after passing through a TSK-GEL G3000SWXL column equilibrated with a buffer containing ATP and other necessary ingredients for exerting its enzymatic activity[9]. The results thus obtained allow the examination of the correlation of the association–dissociation of the subunits with the enzymatic activity. It was found that the minimum unit of the enzyme is the protomer with the composition of $\alpha\beta$

(protomer), and the change in the affinity between a pair of the protomers is sensitive to the ionic environment and temperature. This suggests that the change is directly involved in the expression of the pumping activity of the enzyme.

With membrane proteins, surfactants or lipids are non-covalently bound to them. The technique is also efficient for determining the molar mass of a complex protein to which non-amino acid components are covalently bound. We have shown examples of successful application of the technique for several glycoproteins[10].

Application of the GPC–LALLS technique is not limited to proteins, and we have determined the molar mass of micelles of octaethylene glycol *n*-dodecyl ether[5]. Recently we have applied the technique to the determination of the molar masses of human hepatitis B vaccines, which were produced by the recombinant DNA technique and are protein–lipid complexes with molar mass of several millions[11]. With surfactant micelles, a high sensitivity was required for the LALLS detector but was not necessary for the refractometer. With the vaccine particles, a high performance is required of the refractometer. Temperature control not only of the measuring cells but also of the overall system is required. Temperature fluctuations in any part of the system lead to viscosity fluctuations and thus pressure fluctuations, giving an unstable baseline. In our experience, the measuring system can be used reliably for materials whose molar masses range between several thousands and several millions if the system is fine-tuned. Another example of the application of the GPC–LALLS technique to biological particles is the molar mass determination of protein micelles in milk[12,13]. For flexible polymers with a molar mass comparable to those of vaccine particles and caseine micelles, the reliability may be reduced, however, owing to intramolecular interference of scattered light.

Another application of the technique is the determination of the molar mass distribution of biopolymers. We have successfully determined unequivocally for the first time the molar mass distribution of amylose using this technique[14,15]. Proteins form aggregates when they are heated. When the heating is moderate, some proteins form aggregates that can be fractionated using a GPC column with high porosity. We showed the first example of the observation of such a phenomenon with ovalbumin heated to *ca.* 80°C[16].

In conclusion, we believe that the application of the GPC–LALLS technique in biochemistry has made accurate molar mass measurements of biopolymers easier and has opened up approaches leading to new concepts such as the structure–function relationship of the membrane protein.

Krull's group at Northwestern University is active in the application of GPC–LALLS to the characterization of proteins, and have applied it to detect the gradient elution of proteins from hydrophobic interaction or reverse-phase chromatographic columns[17]. They also showed that detection by LALLS and UV detectors is compatible with an HPLC run in the gradient elution mode. They recently published an excellent review of the application of the LALLS technique to biopolymers[18].

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