Dynamic light scattering (DLS) analyses are routinely used in biology laboratories to detect aggregates in macromolecular solutions, to determine the size of proteins, nucleic acids, and complexes or to monitor the binding of ligands. This article is written for graduate and undergraduate students with access to DLS and for faculty members who wish to incorporate DLS into a lab activity, a practical course or research. It reviews the basic concepts of light scattering measurements and addresses four critical aspects of the analysis and interpretation of DLS results. To ensure reproducible quantitative data, attention should be paid to controlling the preparation and handling of proteins or assemblies because variations in the state of aggregation, induced by minor changes in experimental condition or technique, might compromise DLS results and affect protein activity. Variables like temperature, solvent viscosity, and inter-particle interactions may also influence particle size determination. Every point is illustrated by case studies, including a commercially available albumin, a small RNA virus isolated from plants, as well as four soluble proteins and a ribonucleoprotein assembly purified and characterized by students in the frame of their master degree.

Keywords: Light scattering, protein, temperature, viscosity.

Although the last two decades the panel of methods used to study the structure and function of proteins and nucleic acids has grown continuously. An increasing number of instruments based on physical methods have appeared in biology laboratories. Amongst them, non-invasive spectroscopic methods based on light scattering have become a popular tool in biomolecule characterization. Light scattering measurements have numerous applications in condensed matter physics [1–3], biology [4–7], and medicine [8]. For half a century they have been used to monitor aggregation phenomena in protein solutions. Examples include the study of the influence of various factors on their state of aggregation [9], the detection and monitoring of undesirable aggregates in biotherapeutics that lead to immunogenic reactions or have adverse effects during administration in patients [10], and the investigation of protein aggregation diseases [11], such as caused by interactions between human γD-crystallin molecules with point mutations whose low solubility is responsible for lens opacity during cataract [12].

Owing to intense monochromatic laser light sources, dynamic light scattering (DLS, also known as photon correlation spectroscopy or quasi-elastic light scattering) measurements provide a quick and straightforward means to determine the mutual translational diffusion coefficient (or diffusivity) \( D_t \) of macromolecules in solution. \( D_t \) describes the ease with which a substance displaces inside another one by diffusion and is equal to the mean square displacement of the particles divided by two-fold time [13]. According to Fick’s first law of diffusion, \( D_t \) relates the concentration gradient of a solute
in a solvent along an axis with the flux across a 1 cm² area [14]. It is inversely proportional to particle size, small particles moving faster than large ones.

For samples composed of a single population of particles, two characteristics can be derived from $D_t$. First, the mean hydrodynamic particle size can be calculated assuming a simple geometry like that of a sphere. Second, the polydispersity of the population is given by the standard deviation on $D_t$. DLS is extremely sensitive to the presence of aggregates [15]. In structural biology, it is used to identify solutions in which macromolecules remain monodisperse during crystal nucleation and growth [16–21].

In most universities, the light scattering technology is generally introduced in undergraduate courses in chemistry [22–26] and physics [27] and students get their first “hands on” the experiments. They learn to either analyze the trajectory of spherical particles or determine the molecular weight of water-soluble polymers, the Boltzmann constant and the sub-micron size of colloids or polymer beads. The situation of biology and biomedical students is different because they are rarely taught the principles and have neither an opportunity to see an instrument nor to practice. This is well reflected by the very few publications dealing with introductory laboratory work on biological macromolecules in educational journals [For a static light scattering (SLS) analysis of tobacco mosaic virus, see ref. 28].

This article is based on scientific results obtained by three students during their training for the master degree in biochemistry and molecular biology at the University of Strasbourg, France. It is written as a guide for graduate students who were for the first time in contact with a light scattering instrument. The students cloned the genes of their target biomolecules, overexpressed the latter in Escherichia coli and purified them to homogeneity in the laboratory. Using three different instruments, they performed light scattering measurements on these molecules, as well as on a commercial protein available in greater amounts and on a plant virus in stock. Here we compare two techniques to remove aggregates in biological samples prior to light scattering measurements. We draw experimenters’ attention to the significance of corrections for solvent properties, temperature, and contribution of inter-macromolecular interactions, and discuss why a rigorous comparison of particle sizes is only possible when all measurements are performed at the same temperature. These recommendations should help avoid several pitfalls during the analysis and interpretation of DLS data.

DEFINITIONS AND CONCEPTS

Definitions

A protein solution is composed of a solvent (e.g. water containing a dissolved buffer substance and additives) and a solute (e.g. the protein). A macromolecular solution with a monomodal particle size distribution contains a single population of identical particles. The degree of polydispersity of this solution is essentially due to asymmetry in shape and to intermolecular interactions. In a monodisperse sample all particles are identical in size and shape.

Polydispersity can be very low in the case of spherical particles. It is considered to be satisfactory as long as the standard deviation on $D_t$ or on $d_h$ is $\leq 15\%$. Bimodal solutions containing two populations characterized by intrinsic polydispersities will not be considered here.

The refraction index of a substance is the ratio of the velocity of light in vacuum to the velocity in this substance. That of a solution containing one or several solutes can be considered in first approximation to be equal to the sum of the refractive indices of all chemical bonds.

The viscosity of a liquid is its resistance to deformation or flow. The absolute viscosity (in cP or mPa s) of a substance filling the space between two horizontal planes, is the tangential force per unit area at unit distance apart. The absolute (or dynamic) viscosity required to correct light scattering data, is the product of the kinematic viscosity by the density (or mass/unit volume) of the liquid. Experimentally, kinematic viscosity (in cSt or mm²/s) is derived either from volume of liquid flowing through a tube per unit time under normal gravity or from time taken by a bead to move a given distance through a liquid.

Basics of Light Scattering Theory

Only parts of the theory of light scattering pertaining to the topic of this article are given hereafter. More details can be found in specialized treatises [2–4]. In brief, in a steady aqueous solution maintained at constant temperature, the continuous agitation of water molecules leads to the displacement of the solute molecules dissolved in it. A characteristic of this Brownian motion is that small molecules move faster than large ones. Upon irradiation with visible monochromatic light of high spatial and temporal coherence a small fraction of light is scattered. The intensity of this scattered light fluctuates in a time-dependent manner due to the continuously changing distances between particles. This leads either to constructive or to destructive interference. Intensity fluctuations recorded during a DLS analysis contain information about the time scale of the movement of the scatterers. Their trace can be fitted with an autocorrelation function.

The decay of correlation is directly related to the motion of the particles, their $D_t$, and hence their dimensions. Numerical methods can be applied to extract $D_t$.

Since, protein molecules have dimensions below 1/20 of the wavelength of the light, no angular dependence of the scattered intensity is expected and measurements can be performed at a single scattering angle. In DLS, the time autocorrelation function of the scattered light is used to extract the size distribution of the dissolved particles. The first order electric field correlation function of laser light scattered by a monomodal and monodisperse population of macromolecules can be written as a single exponential of the form

$$G(\tau) = 1 + b \exp(-2Dtq^2\tau)$$  \hspace{1cm} (1)$$

where $b$ is a constant depending on the optics and the geometry of the instrument, $D_t$ the translational diffusion coefficient of the particles, and $\tau$ the characteristic decay
The scattering vector \( q \) is given by
\[
q = \frac{4\pi n_0}{\lambda_0 \sin(\theta/2)},
\]
where \( n_0 \) is the refractive index of the solvent, \( \lambda_0 \) the wavelength in vacuum, and \( \theta \) the scattering angle. For populations composed of a single type of particles, the distribution function of decay rates was derived from a simple fit of the experimental estimates of the logarithm of the correlation function in Eq. (1) to a polynomial. This approach is called the method of cumulants [29]. For polymodal samples, \( G(c) \) is no longer a single exponential and must be represented by an integral over a distribution of normalized decay rates. This data inversion method is known as the regularization method [30, 31]. It gives satisfactory results for bimodal samples composed of particles differing sufficiently in size. Both methods yield close values in the case of monomodal samples.

In the case of hard spheres, \( D_1 \) is related to the hydrodynamic radius \( R_h \) of the particles through the Stokes–Einstein relation
\[
D_1 = k_B T / 6\pi \eta R_h,
\]
where \( k_B \) is Boltzmann’s constant \((1.381 \times 10^{-23} \text{ J/K})\) and \( \eta \) the absolute (or dynamic) viscosity of the solvent [3]. Thus, \( R_h \) is proportional to the inverse of the time relaxation of the decay. In this article, the hydrodynamic diameter \( d_h \) (i.e., \( d_h = 2 \times R_h \)) was preferred to represent particle size. \( d_h \) is given in nm with its standard deviation called polydispersity. Most instrument software display polydispersity in form of a polydispersity index or of a% of polydispersity. The relationship between these three terms is not intuitive and should be verified. Frequently, the percent of polydispersity is equal to the square root of the polydispersity index multiplied by 100.

\( d_{n0} \) is the value of \( d_h \) extrapolated at zero concentration, that is the diameter of freely diffusing molecules in an infinitely diluted solution in which no interaction can occur due to the great distances between particles. In practice, extrapolation from measurements made at higher concentrations is possible only as long as the response of the detection system is proportional to the intensity of the signal.

Interactions between biomolecules in solution cannot be quantified by SLS, also called total intensity, measurements. In practice, this is done by measuring the intensity \( I \) of the light scattered by solutions at various concentrations. \( I \) is a function of particle size and is proportional to particle concentration. Most DLS instruments also measure the average scattering intensity for SLS measurements. The variation of \( I \) can be used to derive the mass of the particles through the Rayleigh equation:
\[
Kc/R_0 = \frac{1}{M} + 2A_2 c \, P(\theta),
\]
where \( R_0 \) is the Rayleigh ratio of scattered light to incident light, \( c \) the concentration (in mg/mL), \( M \) the sample molecular weight (in Da), \( A_2 \) the second virial coefficient characterizing the interactions (in mL mol/g²), \( P(\theta) \) the angular dependence of sample scattering, and \( K \) (in mol cm²/g) an optical constant defined as:
\[
K = \frac{2\pi^2}{\lambda_0^2 n_A} (n_0 dn/dc)^2
\]
with \( N_A \) Avogadro’s number, \( \lambda_0 \) the wavelength of the laser light, \( n_0 \) the refractive index of the solvent, and \( dn/dc \) the increment refractive index with sample concentration (in mL/g).

### MATERIALS AND METHODS

#### Filters

Solvents were filtered on 0.2 μm membranes (Millipore GS units, Cat. No. SLGS033SB) purchased from MilliQ. Protein solutions were filtered on inorganic membrane Anotop 10 Plus filters (diameter 10 mm, pore size 0.02 μm, Cat. No. 6809-0302) from Whatman™.

#### Proteins and Solvents

Bovine serum albumin (BSA) at 2 mg/mL in aqueous 0.9% NaCl solution produced by Pierce was purchased from Thermo Scientific (Cat. No. 23209). Dimeric archael-type nondiscriminating aspartyl-tRNA synthetase from Thermus thermophilus (ttDRS-2, 422 aa, subunit Mr = 48,329, \( E_{280nm} = 1.15 \text{ mg/mL/cm} \)) was produced in E. coli and purified by two chromatography steps after heat-treatment of the extract for 30 min at 70 °C [32]. The pure protein was stored in 20 mM Tris-HCl buffer pH 7.5 [33]. Dimeric archael-type nondiscriminating aspartyl-tRNA synthetase from Deinococcus radiodurans (drDRS-2, 435 aa, subunit Mr = 48,231, \( E_{280 \text{ nm}} = 0.547 \text{ mg/mL/cm} \)) was produced in E. coli and purified by chromatography on DEAE-cellulose, phosphocellulose and hydroxypatite. It was stored at −20 °C in 50 mM Na-Hepes buffer pH 7.2, 0.1 mM Na2EDTA, 5 mM β-mercaptoethanol, and 50% (v/v) glycerol. Dimeric bacterial-type nondiscriminating aspartyl-tRNA synthetase (hpDRS, 577 aa, subunit Mr = 65,601, \( E_{280nm} = 0.471 \text{ mlg/mg/cm} \)) and monomeric glutamyl-tRNA synthetase (hpERS-1, 439 aa, subunit Mr = 51,002, \( E_{280nm} = 0.87 \text{ mg/mL/cm} \)) from Helicobacter pylori were overproduced in E. coli and purified by ion exchange and affinity chromatographies [34]. The transamidosome particle from Thermus thermophilus is a ribonucleoprotein complex composed of AspRS, tRNAAsn, and trimeric TRNA-dependent amidotransferase GatCAB [35] having a mean Mr of 380,000, in agreement with the presence of two dimeric AspRSs (Mr = 100,000), four tRNAAsn molecules (Mr = 25,000 each) and two trimeric GatCABs (Mr = 110,000). This complex was prepared by mixing the components and subsequently purified by size exclusion chromatography (SEC) [36]. Brome mosaic virus (BMV, capsid subunit Mr = 20,385, 180 copies, tripartite ARN Mr = 1.0 × 10⁸, total virion Mr = 4.7 × 10³) is a small RNA virus with a diameter of about 30 nm. It was propagated in barley, purified by precipitation of soluble proteins at pH 4.8 followed by two cycles of low- and high-speed centrifugations. Its concentration was derived from absorbance.
at 260 nm using an extinction coefficient of $\epsilon_{260\text{nm}} = 5.1 \text{ mg/mL/cm}$. For pure solutions $A_{260\text{nm}}/A_{280\text{nm}} = 1.7 \pm 0.1$. Concentrated virus in water containing 0.1% (v/v) sodium azide was filtered through a membrane with a pore diameter of 0.2 μm and stored at 4 °C [37]. Table I lists the biomolecules used in this study together with the composition, viscosity, and refractive index of the respective solvents as well as the temperatures at which experiments were performed.

**Table I**

<table>
<thead>
<tr>
<th>Biomolecule</th>
<th>Solvent composition</th>
<th>$t$ (°C)</th>
<th>$\eta$ (mPa s)</th>
<th>$n_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMV</td>
<td>50 mM Na-Hepes pH 7.5, 10% (v/v) glycerol, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mM Na₂-EDTA</td>
<td>20</td>
<td>1.470</td>
<td>1.351</td>
</tr>
<tr>
<td>BSA</td>
<td>150 mM NaCl</td>
<td>20</td>
<td>1.018</td>
<td>1.332</td>
</tr>
<tr>
<td>drDRS-2</td>
<td>100 mM Tris-HCl pH 8.0, 10% (v/v) glycerol, 5 mM β-mercaptoethanol</td>
<td>20</td>
<td>1.399</td>
<td>1.346</td>
</tr>
<tr>
<td>hpDRS-2</td>
<td>100 mM Na-Hepes pH 7.2, 30 mM KCl, 6 mM MgCl₂, and 5 mM β-mercaptoethanol</td>
<td>12</td>
<td>1.214</td>
<td>1.331</td>
</tr>
<tr>
<td>hpERS-2</td>
<td>50 mM Na-Hepes pH 7.2, 30 mM KCl, 6 mM MgCl₂, 1 mM Na₂-EDTA, 5 mM β-mercaptoethanol</td>
<td>20</td>
<td>1.041</td>
<td>1.335</td>
</tr>
<tr>
<td>Transamidosome</td>
<td>50 mM Na-Hepes pH 7.2, 30 mM KCl, 6 mM MgCl₂, 5 mM β-mercaptoethanol, 0.1 mM Na₂-EDTA</td>
<td>20</td>
<td>1.033</td>
<td>1.335</td>
</tr>
<tr>
<td>ttDRS-2</td>
<td>20 mM Tris-HCl pH 7.5, 50 mM NaCl, 10% (v/v) glycerol, 2 mM MgCl₂, 1 mM Na₂-EDTA, 1 mM DTT</td>
<td>20</td>
<td>1.003</td>
<td>1.330</td>
</tr>
</tbody>
</table>

**Sample Preparation for Measurements**

Prior to DLS measurements, unless otherwise stated, the biological samples were centrifuged (4.5 hour, 10,000 rpm, 11,000 × g) in a refrigerated Sigma tabletop centrifuge or ultracentrifuged (1 hour, 45,000 rpm, 100,000 × g) in a Sorvall Hitachi Discovery M150SE micro-ultracentrifuge.

**Light Scattering Instruments and Analyses**

A DLS instrument is composed of a laser light source, a sample cell, a detector placed at a fixed or variable angle, a photomultiplier amplifying the signal, and a correlator (Fig. 1). Analyses were performed using either of three instruments: a DynaPro™ DP-801 (Protein Solutions, 20 mW He-Ne laser, $\lambda_0 = 780$ nm, scattering angle $\theta = 90°$), a Zetasizer™ NanoS (Malvern Instruments, 4 mW He-Ne laser, $\lambda_0 = 633$ nm, $\theta = 173°$) and a NanoStar™ (Wyatt Technologies, 100 mW He-Ne laser, $\lambda_0 = 633$ nm, $\theta = 90°$). Corresponding quartz cells were filled with 12, 20, or 2 μL sample solution. The cells, placed pairwise on cushions of soft paper inside the round-bottom tube holders of the six-place angle rotor of a small Sigma 1-6P bench centrifuge, were centrifuged for 10 min at 1,500 × g to eliminate air bubbles. Before a cell was introduced into the instrument, its outer surfaces were wiped gently with a sheet of soft lens cushions of soft paper inside the round-bottom tube holders of the Discovery M150SE micro-ultracentrifuge.

**Other Analytical Methods**

Refractive indices of solvents (i.e., the buffer solutions without protein) were measured on 20 μL sample solution with a student Abbe refractometer kept at 20 °C in an air-conditioned room. Solvent densities (in g/cm³) were determined at 20 °C from mass of a 5-mL glass pycnometer, either empty or filled with water (density $= 1$ g/cm³) or with solvent. Solvent kinematic viscosities were determined by two methods. Micro-Ubbelohde capillary viscosimeter tubes (volume 3.5 mL, Schott, Germany) immersed in vertical position in a water bath at constant temperature, were used to measure the time taken by the upper meniscus of the solution to travel between two fixed points. To minimize the experimental error, the diameter of the capillary was chosen so that the time measured with a handheld stopwatch was ~ 100 second. This time was compared to that of pure water and kinematic viscosity (in mm²/s) was derived by applying a capillary-dependent correction factor.

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### FIG. 1.

Schematic representation of the optical setup of a DLS system. By convention the scattering angle $\theta$ is the angle between the incident beam coming out of the sample and the detector. An angle of 90° is a good compromise for the analysis of protein and virus samples, which have a size that is $\leq 20$. $\theta$ can be as great as 173° for backscattering measurements. Modern instruments have a second detector measuring the total scattering intensity for SLS calculations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Absolute viscosity (in mPa s) is the product of kinematic viscosity by density. Other measurements were performed using an AMVn falling-bead viscosimeter (Anton Paar, Austria) equipped with a 150 µL temperature-controlled glass capillary calibrated with water. The time needed for a steel bead of known density to travel a fixed distance within a tube tilted by a given angle at constant temperature was recorded to determine the kinematic viscosity. In our hands, the first method was more delicate and time-consuming. In the absence of above instruments, databases—established and maintained by specialists of analytical ultracentrifugation or by light scattering instrument manufacturers—can be consulted to find the properties of a variety of compounds. Dedicated algorithms calculate the properties of complex mixtures (e.g., SEDNTERP; see [38, 39]).

**Safety**

In principle, the laser source contained inside the light scattering instrument poses no health hazard even when the lid of the apparatus is open. However, adapted eye protection is required when working with a laser source installed on an optical bench where the light beam travels through the air. To avoid skin and eye contact with the irritating solutions used to clean quartz cells, experimenters should wear latex or nitrile gloves and eye protection.

**Experimental Errors**

The error on solvent density was <0.1% when using a standard laboratory weighing balance operating between 1 and 100 g with a precision <1 mg. That on refractive index was estimated to 0.1% for a series of 10 readings in the 1.333–1.350 range done with a student Abbe refractometer. It was 1% (i.e. 1 second on a fall time of 100 second) for triplicate kinematic viscosity measurements using thermostated fall-bead viscosimeters and 5% for a glass capillary viscometer, due to the addition of errors on time and temperature.

For DLS measurements, the error on particle diameter was due to that on viscosity and on refractive index when data were extrapolated to zero concentration. For SLS measurements, the major source of error on mean particle mass was the error on particle concentration when populations had monomodal distributions and were monodisperse.

**RESULTS AND DISCUSSION**

The most recent high-performance, small-sized DLS instruments found in biology laboratories do automated measurements on a few microliters of dilute solutions of small particles and user-friendly algorithms analyze and interpret data instantaneously. With the decreasing involvement of the user there is an increasing danger that inexperienced newcomers ignoring elementary concepts do not respect basic rules and misinterpret their results. As shown here by the results obtained by the students, first and foremost, the biological samples should be prepared in a standardized way to ensure reproducible results. Large aggregates should be removed systematically, except when the purpose of the experiment is to detect them. Second, for a single population of particles, $D_i$ is only meaningful if solvent viscosity and refractive index are taken into account. These corrections are not outdated and should neither be underestimated, overlooked nor bypassed. Third, interactions between particles are seldom negligible. Values of $D_i$ or of particle size that are independent of them should be derived from measured data by extrapolating $D_i$ or particle size to zero protein concentration, as in the past [22]. Finally, the parameter temperature should not be neglected because particle size distributions may be temperature dependent.

**Preparation and Handling of Monodisperse Biomolecules**

When the students analyzed their samples by DLS, they were rapidly convinced that the absence of aggregates in pure macromolecular samples (i.e. containing ≥95% target molecule) is a sign of high quality and a premise to good measurements. Aggregation is frequent in protein solutions under oxidative conditions resulting from contact with air upon strong shaking [40] as well as after cycles of freezing-thawing in the absence of cryoprotectant hindering the formation of ice crystals or after freeze-drying that removes bound water molecules. Other possible causes are insufficient or too high ionic strength, the presence of divalent or trivalent ions establishing ionic interactions between molecules, hydrophobic interactions or a pH too close or far from isoelectric point. When aggregates are present in the samples, various remedies may be successful to dissolve them, including the addition of a reducing agent [e.g. 1–5 mM β-mercaptoethanol, dithioerythritol, dithiothreitol, Tris(2-carboxyethyl) phosphine], a moderate concentration (e.g. 150 mM) of a monovalent salt, a low concentration of mild nonionic detergent (e.g. below critical micelization concentration) or organic solvent (e.g. 1% v/v isopropanol), as well as 50% (v/v) glycerol for storage at −20°C. Coenzymes, substrates or analogs may also increase macromolecular stability and solubility.

The students work suggested that the determination of a hydrodynamic particle size by DLS can be more accurate when samples have monomodal size distributions and are monodisperse. For this reason, unstructured aggregates that contaminate highly pure molecules had to be removed prior to measurements. Such impurities were eliminated by either of two simple techniques, filtration or centrifugation. Filter membranes with the appropriate cut-off separated rapidly the target from unwanted particles, but filtration had limitations. In the case of the aminoacyl–tRNA synthetases prepared by the students, microliter sample volumes could hardly be filtered and there was a risk that a variable amount of biological material may adsorb onto the membrane or the prefilter. For the purpose of teaching an experiment was done on commercial BSA. Figure 2 shows the effect of filtration through a membrane with pores of a diameter of 20 nm on the homogeneity of this protein. It removed aggregates with greater dimensions that contributed ~5% of the intensity but represented less than 1% of the protein mass. This low populated state was detected because the intensity of the scattered light is proportional to the sixth power of the particle diameter. In the present case, the amount of aggregates was too low to perturb the determination of the size of BSA particles. Particle size determination becomes delicate when heterogeneous
populations of aggregates dominate in solution and contribute more than 50% of the intensity (result not shown).

In SLS measurements done at a single angle, the supplementary scattering intensity produced by aggregates leads to an overestimation of the particle mass derived from Debye plot. In MALS experiments, this problem is overcome by separating aggregates from target molecule on a SEC column or by field flow fractionation prior to measurements. However, this does not reduce the heterogeneity of the latter.

Centrifugation was an interesting alternative to filtration for biomolecules binding to filter membranes (e.g. nucleic acids and possibly nucleoprotein complexes in which negatively-charged phosphate groups of the nucleic acid are exposed to the solvent) or for which only small volumes are available (as in the case of the enzymes purified by the students). The samples can be subjected to a wide range of accelerations and a broad range of volumes can be treated, from hundred milliliters down to a few microliters. Figure 3 shows the effect of centrifugation on the homogeneity of soluble drDRS-2. The soluble protein was free of aggregates, monomodal, and less polydisperse. The students also observed that sometimes aggregates formed when concentrated protein solutions were diluted or transferred into the quartz cells for the DLS measurement. Aggregate formation was reduced when the quartz or polymer surfaces of sample cells were wetted with a thin film of buffer solution before proteins were in contact with them.

**Importance of Viscosity Correction for Size Determination**

During their experiments, students raised the question whether the composition of the solvent (e.g. the presence of a substantial concentration of glycerol) influences the behavior of the proteins. As it can be seen in Eq. (3), the rate at which particles displace in solution by Brownian motion is inversely proportional to the viscosity of the medium. For a given particle, the values of \( D_t \) determined in solvents with different viscosities and refractive indices must be corrected before they can be compared. The viscosity, refractive index, and density of aqueous solutions vary with temperature as those of pure water. In principle, the properties of any solution can be derived from its composition if the contribution of every ingredient is known. However, as mentioned, many proteins require for their stability the presence of chemicals (e.g. reducing agents or detergents) or biochemicals (e.g. ligands-like coenzymes or substrates). In the absence of information about the viscosity or refractive index of some of these substances, it is preferable to determine experimentally the real characteristics of every solvent used in light scattering analyses. SLS data require to be corrected only by the increment of refractive index of the particles.

As it can be seen in Table II, the refractive index, density, and volume expansion of water vary by no more than 0.1% per interval of 5°C between 15 and 25°C. A simple student Abbe refractometer, an inexpensive 5-mL pycnometer, and a laboratory balance were sufficient to determine these parameters with good accuracy and precision. At variance, the viscosity of water varies by ~25% between 15 and 25°C and temperature had to be
Table II

<table>
<thead>
<tr>
<th>Properties of water near room temperature</th>
<th>Temperature (°C)</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>% Variation per 5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molar refractive index&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.33341</td>
<td>1.33299</td>
<td>1.33251</td>
<td>0.03/0.04</td>
<td></td>
</tr>
<tr>
<td>Absolute viscosity (cP)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.139</td>
<td>1.002</td>
<td>0.8904</td>
<td>13.6/12.5</td>
<td></td>
</tr>
<tr>
<td>Density (g/ml)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.99913</td>
<td>0.99823</td>
<td>0.99707</td>
<td>0.09/0.11</td>
<td></td>
</tr>
<tr>
<td>Volume&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.0031</td>
<td>1.0041</td>
<td>1.0052</td>
<td>0.1/0.1</td>
<td></td>
</tr>
</tbody>
</table>

All data from ref. 41.

<sup>a</sup> Variation is not a linear function.

<sup>b</sup> Relative to air.

<sup>c</sup> Absolute viscosity = kinematic viscosity × density.

<sup>d</sup> Expansion calculated using the relationship \( V_1 = V_0 (1 + \beta t) \) with \( V_0 \) the volume at 0°C, \( \beta \) the coefficient of thermal expansion (\( \beta = 2.07 \times 10^{-4} \) per °C), and \( t \) the temperature in °C.

controlled carefully to get reliable measurements. This was done either by immersing the glass viscosimeter tubes in a thermostated bath or by using a falling-bead viscosimeter equipped with Peltier elements. The later required a smaller volume of buffer (and not protein) solution and less time.

Figure 4 shows the case of hpDRS in a solvent for which the corrections were negligible. In other words, the effects of viscosity and refractive index compensated.

Effect of Interactions on Apparent Particle Size

The inexperienced students were surprised that interactions can affect the result of a size determination and that a false impression of greater or smaller particle size may result from the concentration dependence of \( D_r \). Figure 6 displays three examples that illustrate this point well. The upper plot in the left panel shows that the \( d_h \) of ttDRS-2 increased linearly between 1 and 20 mg/mL. Assuming that particles were globular, their mass augmented from \( \sim 150,000 \) to \( \sim 450,000 \) over this concentration range. In other words, dimeric ttDRS-2 behaved as a dimer at 1 mg/mL and as a trimer of dimers at 20 mg/mL. A 60% increase in \( d_h \) and the deduced threefold increase in mass were only apparent. They were due to attractive interactions between particles in solution. The concentration dependence of \( D_r \) was not negligible. Extrapolation of \( d_h \) to zero concentration suppressed the effect and gave the correct \( d_h \).

The two plots in the left panel of Fig. 6 demonstrate with ttDRS-2 that the strength of protein–protein interactions depended upon the composition of the solvent. On the one hand, it was shown elsewhere that glycerol increases the solubility of proteins by replacing water molecules at their surface [42]. On the other hand, small ions like Na<sup>+</sup> and Cl<sup>−</sup> are known to produce a “salting in” effect by shielding charges. Interestingly, the opposite effect was observed within a limited range of biomole-

This sample could have been analyzed without knowing these parameters as if it were in pure water. This peculiar situation occurred sometimes with buffer solutions containing salts (like KCl) having negative viscosity increments.

However, this is a case in isolation. Figure 5 juxtaposes the strong effects observed with hpDRS and BMV when the buffer was either cooled (Fig. 5a) or made more viscous (Fig. 5b), respectively. In both cases, the particles seemed to have smaller diffusion coefficients, and accordingly greater hydrodynamic diameters. Appropriate corrections shifted the size distributions towards smaller diameters without modifying polydispersity, which is an intrinsic property of the sample.
cule concentration and in the presence of some compounds. Then, the apparent particle size seemed to diminish as a consequence of repulsive interactions. The phenomenon observed in dilute solutions of the ribonuclease complex transamidosome is displayed in the right panel of Fig. 6. As in the case of attractive interactions, extrapolation of \( d_1 \) to zero concentration suppressed the effect and gave the correct \( D_1 \) and \( d_2 \). The diameter found by DLS was in agreement with the one determined by small-angle X-ray scattering (SAXS) [43].

Intermacromolecular forces involve hard core, short-range attractive van der Waals and repulsive electrostatic coulombic interactions. They can be quantified using SLS, SAXS, or osmotic pressure measurements [44]. Their nature is reflected by the sign of the second virial coefficient, positive \( A_2 \) values being characteristic for attractive potentials. Their strength is indicated by how much \( A_2 \) departs from zero. Table III lists the \( A_2 \) values of several proteins determined at 20°C in various buffer solutions.

**Table III**

<table>
<thead>
<tr>
<th>Biomolecule (source)</th>
<th>Mr</th>
<th>Solvent composition</th>
<th>( t ) (°C)</th>
<th>( A_2 \times 10^4 ) (ml mol/g²)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme (hen egg-white)</td>
<td>14,313</td>
<td>50 mM Na-acetate pH 4.5</td>
<td>20</td>
<td>+1.0</td>
<td>[45]</td>
</tr>
<tr>
<td>Urate oxidase (Aspergillus)</td>
<td>136,440</td>
<td>50 mM Na-borate pH 8.7</td>
<td>20</td>
<td>+0.24</td>
<td>[46]</td>
</tr>
<tr>
<td>Transamidosome (Thermus th.)</td>
<td>380,000</td>
<td>50 mM Na-Hepes pH 7.2, 6 mM MgCl₂, 30 mM KCl, 5 mM β-mercaptoethanol, 0.1 mM Na₂EDTA</td>
<td>20</td>
<td>0 ± 10</td>
<td>[35]</td>
</tr>
<tr>
<td>γ-Crystallins (calf lens)</td>
<td>20,965</td>
<td>10 mM NaK phosphate pH 7.0, 5 mM DTT</td>
<td>20</td>
<td>−0.4</td>
<td>[37]</td>
</tr>
<tr>
<td>Brome Mosaic Virus</td>
<td>4.6 × 10⁶</td>
<td>20 mM Na-acetate pH 5.9</td>
<td>20</td>
<td>+0.026 ± 0.005</td>
<td>[47]</td>
</tr>
<tr>
<td>Tomato Bushy Stunt Virus</td>
<td>8.8 × 10⁶</td>
<td>50 mM Na-acetate pH 4.5</td>
<td>20</td>
<td>−1.5 ± 0.5</td>
<td>[37]</td>
</tr>
</tbody>
</table>

**Effect of Temperature on Apparent and on Real Particle Size**

Two effects must be distinguished. The first one was rapidly accepted by the students who knew that water becomes less viscous when temperature increases. Solvent viscosity and refractive index corrections are essential for a rigorous comparison of DLS results obtained at different temperatures. The effect of temperature on particle size can also be investigated using various separation methods (like SEC, electrophoresis, and isoelectric focusing under native conditions, and analytical ultracentrifugation), analytical methods measuring scattering properties (e.g. SAXS), binding (like isothermal calorimetry and surface plasmon resonance), or enzymatic catalysis assays. The plots in the panels of Fig. 7 show that the hydrodynamic diameters of the transamidosome and of drDRS-2 actually did not vary significantly once the corrections for viscosity variation with temperature were applied.

The second effect is due to temperature-dependent protein aggregation. For example, the enzyme ttDRS-2 began to precipitate when solutions at >2.5 mg/mL in 20 mM Tris-HCl buffer pH 7.5 were cooled, for example when samples at room temperature were placed on ice. A 14 mg/mL solution showed a milky aspect but cleared off as soon as it was held in the hand. As a consequence, part of the protein sedimented during centrifugation at low temperature. The graphical representation of DLS results in Fig. 8 provided an explanation of the observed phenomenon. The \( d_i \) of the protein increased strongly below the critical temperature of 5°C, intermolecular interactions were stronger at lower temperature (i.e. the binding energy increased), and association was reversible. At the other end of the temperature scale, the \( d_i \) became gradually slightly greater, probably as a consequence of a more relaxed state of the protein molecules before they unfold and aggregate at around 70°C (result not shown). The latter phenomenon was reversible as long as temperature was below 51°C. This correlated well with the inflexion point at ~ 50°C seen in the biphasic Arrhenius plot [48]. Finally, salts and organic osmolytes, like glycerol, were shown to modulate protein stability and solubility [49].

**DLS Versus Other Analytical Methods**

The DLS method is user-friendly and requires little sample handling. The measurements need only small...
sample volumes, take only a few minutes per sample, and are not invasive. The minimal sample concentration depends upon the power of the laser and as little as 1 or 2 µL is enough to perform measurements in the most sensitive instruments. The sample is placed in an open cell whose temperature is well controlled and it is irradiated with a beam of visible light (the wavelength of the laser light should be adapted for colored proteins). After the measurement, the sample can easily be recovered for other analyses. Titration experiments can be performed in this cell simply by adding stepwise small volumes (e.g. 0.5 or 1 µL) of concentrated additive solution to a volume of protein solution (e.g. 20 µL) and by monitoring the variation of the mean particle diameter as a function of additive stoichiometry.

As illustrated above with protein solutions, DLS measurements are extremely useful to detect very small amounts of larger particles. Information about the size and quantity of such aggregates can be derived from the particle size distributions as a function of intensity, volume, number, or mass computed by the software of most instrument manufacturers. These objects can also be characterized using SEC (except when shearing forces dissociate them) and analytical ultracentrifugation [50]. This may be of importance when activity of the protein or nucleoprotein complex is affected by variations in the state of aggregation that might be induced by minor changes in experimental conditions or of experimental technique.

For monomodal and monodisperse protein samples, DLS measurements provide information on particle size, which is complementary to that coming from other analytical methods, like SEC, electrophoresis, mass spectrometry, analytical ultracentrifugation, or SAXS. Analyses under native or dissociating conditions are necessary to deduce separately information on the size of the monomeric entity and that of the oligomeric state. In DLS, the measurement is carried out on particles on that may be in dynamic equilibrium and the sample is not separated in its components. At variance, SEC, analytical ultracentrifugation or free flow fractionation sort proteins according to their hydrodynamic properties [51] and mass spectrometry according to mass or charge. The latter method can be useful to investigate the composition of intact and dissociated protein complexes [52, 53]. Electrophoresis separates proteins according to size, shape, and charge under native conditions and polypeptide chains separate essentially according to their bulkiness (i.e. mass and volume) and their charge under denaturing conditions.

A diffusion coefficient equivalent to the one determined by DLS can be calculated from SEC data, by comparing the elution volume of an unknown sample to that of proteins with similar shapes and known hydrodynamic diameter. $D_t$ can also be obtained from an equilibrium ultracentrifugation experiment. SAXS measurements give the radius of gyration $R_g$, equal to 0.774 $R_h$ for globular particles. The results of all above methods may be more difficult to interpret when the protein is solubilized with a detergent, because the solution then contains detergent monomers, pure micelles and micelles containing protein that scatter light. These micelle populations can be resolved by DLS only if they differ enough in size.

**FIG. 7.** Particles with minor temperature-dependent diameter variations. (Left panel) Variation of the particle mean hydrodynamic diameter of transamidosome particles in a solution at 1 mg/mL analyzed between 4 and 55°C. The highest temperature was 15°C below the denaturation temperature of the complex ($T_{dn} = 71 ± 1°C$ in this solvent). The DynaPro TM DP801 data were corrected at every temperature for solvent viscosity and refractive index. The data extrapolate at $d_h = 13.4 ± 3.3$ nm for $T = 0°C$, $d_r$ and polydispersity (~25%) did not vary very much with temperature. When temperature approached 71°C they increased dramatically as a consequence of the unfolding and aggregation of the protein and nucleic acid composing the particles (result not shown). (Right panel) Variation of the particle mean hydrodynamic diameter of dDRS-2 at 0.8 mg/mL. The Zetasizer TM NanoS data were corrected for solvent properties at every temperature. The data extrapolate at $d_h = 8.7 ± 2$ nm for $T = 0°C$. Polydispersity (~18%) did not vary. All data are displayed with a 5% error bars. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**FIG. 8.** Variation of the protein hydrodynamic diameter with temperature. The sample contained 2.5 mg/mL ttDRS-2 in 20 mM Tris-HCl pH 7.5 ($n_1 = 1.003$ mPa s, $n = 1.330$). The DynaPro DP801 data corrected for buffer properties at every temperature are displayed with 5% error bars. (Upper plot) Temperature was first increased from 20 to 50°C by steps of 5°C, then decreased to 0°C, and finally increased back to 20°C (as indicated by arrows) by steps of 5°C except between 10 and 0°C where the intervals were 3°C. The experiment was pursued neither below 0°C to prevent the protein solution from freezing nor above 50°C to avoid protein denaturation. All measurements were done after 5 min equilibration time. (Lower plot) Sample polydispersity (in nm) was not much affected by temperature. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
The major limitation of DLS is that particle populations differing by less than a factor 3 in diameter cannot be resolved. This is a hindrance when one wants to identify a complex that is not much greater in size than the initial protein. Comparison may then be possible only by overlaying the size distributions of both particles analyzed independently in a pure state. Another limitation is that the light scattered by a small number of aggregates can mask more interesting components and compromise seriously the results when the sample has to be analyzed without any treatment like filtration or centrifugation.

CONCLUSION

This article, reporting the work done by graduate students during their master degree, has examined various practical aspects of the use of DLS for the characterization of proteins. The experimental results demonstrated that homogeneous samples containing single populations of particles, all identical in size and shape, were the sinea non condition for optimal data analysis and interpretation. Undesirable aggregates had to be removed prior to measurements. They confirmed that the interpretation of the results of DLS measurements must take into account the properties of the medium in which the biomolecules were dissolved. In rare cases, the solvent properties were so close to those of water that corrections were superfluous or their oversight passed unnoticed. In all other cases, failure to apply appropriate corrections inevitably led to incorrect diffusion coefficients, erroneous particle dimensions and quaternary structures. The effect was strongest when the viscosity and the refractive index of the solvent differed too much from those of water. An automated viscosimeter was a profitable investment that helped obviate the risk of data misinterpretation. Routine analyzes were accelerated by including corrections in operating procedures. Otherwise, data were corrected afterwards when solvent properties were not available at the time of the measurements.

Another necessary condition for a better interpretation of DLS results was that diffusion coefficients and particle sizes were those of particles in infinitely dilute solutions where interactions are negligible. Such data were more reliable and appropriate for comparison. It required only a small series of measurements at various concentrations to establish a first plot and get an idea of the importance of interactions. If needed, the interactions may be quantified more accurately by determining second virial coefficients using SLS measurements.

Finally, the analyses performed by the students showed that temperature played an important role in all light scattering measurements. $D_v$ values and the derived particle sizes were realistic only when temperature was taken into account. The comparison of light scattering data with data coming from other measurements (e.g. thermodynamic binding parameters or biological activities), was only meaningful when all were obtained at the same temperature. Differences in experiment temperatures of less than 10 °C were great enough to make interpretation difficult or end in contradictions.

The few rules given here are simple to put in practice. They should help strengthen the light scattering data obtained by students as well as other investigators and simplify the determination of protein sizes in solution.

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