

Rayleigh scattering technique as a method to study protein–protein interaction using spectrofluorimeters

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Rayleigh scattering intensity of a protein solution can be measured using a spectrofluorimeter when its excitation and emission wavelengths coincide. Such scattering intensity from proteins having no chromophoric groups in the visible region has been studied at 350 nm. Linear dependence of scattering intensity with molecular weight and concentration of proteins has been observed according to the modified Debye–Zimm relation. In the absence of self-aggregation, scattering intensity observed from non-interacting proteins was additive. Assuming that globular proteins are spheres, analysis using Stoke's radii of the proteins predicted that the change in scattering intensity during association–dissociation of homo multimeric proteins will be at most $\pm 10\%$. This has been confirmed from experimental data. However, significant deviation from additive values of scattering intensity was observed among interacting proteins of unequal sizes as the character of the solution was changed from 'ideal' to 'non-ideal'. The protocol may be applied to evaluate different parameters of protein–protein interaction.

Keywords: Protein–protein interaction, Rayleigh scattering, spectrofluorimeter, Stoke's radius.

MONITORING protein–protein interactions by static and dynamic light scattering is a well-developed subject^{1–7}. Dedicated instruments are available that can measure scattering intensity at variable angles yielding varieties of physical data of the test samples. Notwithstanding the analytical power of static and dynamic light scattering photometers, spectrofluorimeters are also used to measure Rayleigh scattering intensity at 90° by fixing excitation and emission at the same wavelength. The advantage of using spectrofluorimeters is that the instruments are readily available and the results are easy to interpret, though the information gained might be limited. Earlier, an increase of such scattering intensity from soluble proteins, that was measured by spectrofluorimeters was qualitatively related to aggregate formation, e.g. folding of lactic dehydrogenase⁸, thermal unfolding of glutamine synthetase⁹, folding of UDP-galactose 4-epimerase from *Escherichia*

*coli*¹⁰, etc. Later, existence of a dimeric form of bovine serum albumin (BSA) at low pH was predicted using scattering intensity data from a spectrofluorimeter. While calculating molecularity of the conformer, it was assumed that BSA molecules are solid spheres and they touch each other at a single point like marbles. Molecularity of the conformer was confirmed by independent and absolute methods like analytical ultracentrifuge¹¹. Interaction between adenosine kinase (39 kDa) and cyclophilin (19 kDa) was explained from Rayleigh scattering intensity assuming that when two molecules are not interacting, contribution to Rayleigh scattering intensity by the components of the solution will be additive¹². Alternately, any deviation from the additive value should indicate a positive interaction. In the meanwhile, this scattering mode was applied for phospholipase A₂ enzyme assay¹³ and to follow interaction between lysozyme and human placental extract used as drug¹⁴. However, all these assumptions need support from theoretical as well as experimental verification.

The objective of the present study is to establish that the fundamental rules of Rayleigh scattering are faithfully obeyed when a spectrofluorimeter is used. Thereafter, the scope and limitations of such scattering studies in protein–protein interactions of homogeneous ($nM \rightleftharpoons M_n$) and heterogeneous ($N + M \rightleftharpoons NM$) systems have been described. It should be noted that the scattering intensity as referred to in this case is from clear and transparent solutions and is different from turbidity. Turbid particles are bigger, out of phase and show absorption at visible wavelength, irrespective of having chromophores.

Theory

A modified form of the Debye–Zimm relation best represents the scattering intensity from globular proteins up to around 100 kDa and at UV–VIS range of irradiation⁷. In brief, the basic equation is:

$$Kc/R_\theta = \{1 + (16\pi^2 R_g^2/3\lambda^2)\sin^2(\theta/2)\}\{(1/M_r) + 2Bc\}, \quad (1)$$

where R_θ is the Rayleigh scattering intensity normalized with respect to the incident light intensity, c the protein

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concentration, K an experimental constant dependent on solvent refractive index, R_g the radius of gyration, M_r the weight average molecular weight of the protein, λ the wavelength of irradiation or scattering, θ the angle of scattering and B a virial coefficient. Under the experimental conditions, the following assumptions have been made: at very low concentration of protein, $Bc \rightarrow 0$ and the term $16\pi^2 R_g^2/3\lambda^2$ attains negligible numerical values; for example, this term reduces to 0.00047, 0.00045, 0.0010 and 0.0019 for ribonuclease, α -lactalbumin, β -lactoglobulin and bovine serum albumin respectively¹². For a spectrofluorimeter where $\theta = 90^\circ$, $\sin^2(\theta/2)$ becomes 0.5. Thus, the whole term within the first second bracket is essentially reduced to 1. So, eq. (1) is simplified to:

$$R_\theta = KcM_r \quad (2)$$

Assuming that globular proteins are spherical, Rayleigh scattering intensity will ultimately depend on Stoke's radius as:

$$R_\theta = Kc(4/3)\pi r_s^3 \rho, \quad (3)$$

where r_s is the Stoke's radius and ρ the partial specific volume of globular proteins which is considered as a constant. That the molecular weight (Mw) of proteins is linearly dependent on the third power of Stoke's radii has been verified earlier in cases of monomeric ($n = 18$), dimeric ($n = 12$) and tetrameric proteins ($n = 13$)¹⁵. The values of Stokes radii and Mw for this correlation have been taken from elsewhere^{16,17}.

Thus eq. (3) is reduced to:

$$R_\theta = K'cr_s^3, \quad (4)$$

where K' is a new constant.

Stoke's radius of proteins of known molecular weight was derived from Uversky's relation (eq. (5)), when it is not available in the literature¹⁸. This empirical relation is firmly based on experimental evidences and has been applied in protein-folding studies^{19,20}.

$$\log(r_s) = -(0.254 \pm 0.002) \pm (0.369 \pm 0.001)\log(\text{molecular mass}). \quad (5)$$

When two or more spherical proteins interact in stoichiometric proportions, it is assumed that either they melt to form a bigger sphere of combined molecular weight or come in contact at a point without changing individual geometry. In reality, the situation lies in between. Since stable interaction between two proteins or subunits of proteins requires extended overlapping, the second assumption has not been considered here. Stoke's radii of a multimer or a protein complex have been predicted from Uversky's relation eq. (5).

Materials and methods

Reagents

Standard proteins, e.g. alcohol dehydrogenase, bovine serum albumin (BSA), catalase, cytochrome *c*, haemoglobin, lysozyme, ovalbumin, trypsin, soybean trypsin inhibitor (STI) and organic molecules, e.g. benzedine dihydrochloride, thioridazin, verapamil and 2-mercaptoethanol (2-ME) were from Sigma. Urea (E. Merck, India), glycerol (BDH, Mumbai), sucrose (SRL, Mumbai) and all other reagents were of analytical grade. Buffers of 50 mM salt concentration were used as follows: Na-acetate (pH 6.0 and below), Na-phosphate (between pH 6.0 and 8.0) and Tris, HCl (pH 8.0 and above).

Preparation of samples

All test samples were centrifuged at 10,000 rpm for 2 min followed by passing through 0.45 μ m nylon filter membranes (Millipore). Cleaning of glassware was done as recommended¹. To minimize dust contamination from turbulence, air circulation was suspended while transferring samples. In spite of precautions, complete removal of dust was not possible. Sometimes the dust particles appeared as spikes in the recording, but did not interfere with the overall experiments. In case a recording was too noisy, the sample was replaced by a new one.

Measurement of scattering intensity

Scattering intensity measurements were done with a spectrofluorimeter (Hitachi F 4500 or F 3010) attached to a constant temperature circulating water bath (25°C, Polyscience, USA). A 3 ml quartz cuvette with Teflon cover was used. A minimum volume of 1 ml of sample was used. Unless mentioned, excitation and emission wavelengths and slit widths were 350 and 2.5 nm respectively, for both the windows. For a single datum, scattering intensity was recorded for 300 s and the minimum intensity was considered. This eliminated interference from particulate matter. While following kinetics of a reaction from increase or decrease of scattering intensity, the curve generated was smoothened by the inbuilt programming of the instrument. In all sets, the minimum and not the average scattering intensity was noted. It may be mentioned that in the case of scattering from a laser source, the variation of intensity originated from the entry and exit of particles to or from a tiny volume of irradiation that is controlled by thermal diffusion. Therefore, averaging of the data is required²¹. In the present protocol, the volume of sample irradiated being large, the number of particles in that volume is statistically constant.

Dissociation of multimeric proteins

Epimerase from both yeast and *E. coli* (1 mg/ml) was dissociated after incubation in low-salt²² buffer (1 mM Na-phosphate, pH 7.0) for 16 h at 25°C. Catalase (2 mg/ml) in 0.5 M Na-phosphate, pH 7.5, was succinylated at 25°C by adding succinic anhydride in small aliquots from a stock of 1 mg/ml in water, over 30 min under stirring condition²³. It was ensured that the pH of the solution remained constant during the reaction. Alcohol dehydrogenase (1 mg/ml) at pH 7.0 was diluted 20 times to pH 4.0, where the protein was dissociated into its subunits. Glucose-6-phosphate dehydrogenase (1 mg/ml) was dissociated²⁴ by heating at 45–46°C. β -Lactoglobulin (1.4 mg/ml) and hexokinase (1.5 mg/ml) were dissociated at pH 2.6 and 7.0 respectively^{25,26}. Removal of excess reagent or re-equilibration of buffer was done by dialysis or using Sephadex G-25 'spin column'²⁷.

Denaturation of proteins

Proteins (1 mg/ml) were dissolved in 20 mM Na-phosphate, pH 7.5, containing 8 M urea or 6 M guanidinium, HCl and was incubated at 37°C for 2 h in the presence or absence of 10 mM 2-mercaptoethanol (2-MC). It is assumed that equilibrium unfolded structures of proteins are attained under these conditions.

Results and discussion*Scattering from proteins*

Rayleigh scattering being inversely proportional to the fourth power of the wavelength of irradiation, it increases from 800 to 300 nm. For proteins, the rise is followed by a sharp drop due to strong absorption of aromatic amino acids in the near-UV region¹. The profile of scattering intensity of BSA between 280 and 800 nm is in tune with its absorption profile (Figure 1). Compromising scattering intensity with absorption, all scattering intensity measurements, as presented here, were done at 350 nm. At this wavelength, absorption of proteins devoid of visible chromophores is zero. Linear dependence of scattering intensity of BSA against 0–500 μ g/ml of concentration was observed (Figure 1). These spectral features were alike for other reference proteins like lysozyme, ovalbumin and alcohol dehydrogenase. For proteins containing cofactors or prosthetic groups absorbing in the visible region, their scattering intensity profiles were different. Scattering was completely quenched at 405 nm for haemoglobin, cytochrome *c* and myoglobin that corresponded to the 'Soret peak' of the in-built haeme group at 410 nm²⁸ (data not shown). Such proteins were debarred from this study.

To ascertain contributions of buffer components towards scattering intensity, the following ligands were tested whose Mw, concentration and scattering intensity respectively, have been mentioned in the parenthesis: distilled water (18, 100%, 27); 50 mM K-phosphate, pH 7.5 termed as buffer (136, 20 mM, 45), buffer with benzedine dihydrochloride (257, 420 mM, 57), buffer with thioridazin (407, 140 mM, 107) and buffer with verapamil (491, 150 mM, 67). Since Mw and concentration of the ligands

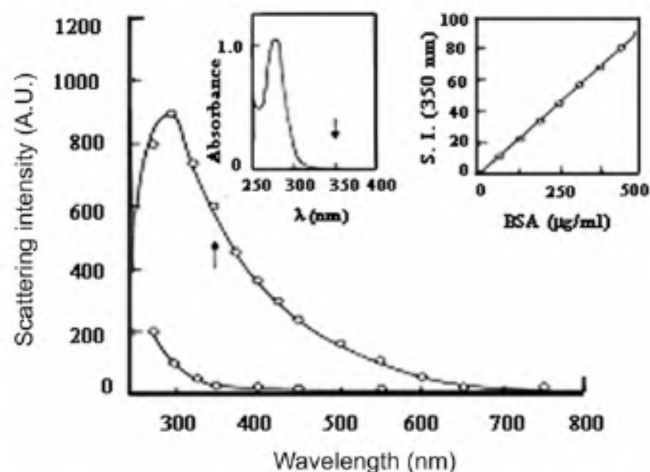


Figure 1. Dependence of scattering intensity of BSA (0.15 mg/ml in 20 mM Na-phosphate, pH 7.5) with wavelength. The lower tracing indicates scattering intensity from buffer and has not been corrected from the sample profile. The upward arrow indicates intensity at 350 nm. (Inset, left) Near-UV absorption spectra of BSA. The arrow indicates that at 350 nm, absorption of the protein is essentially zero. (Inset, right) Linear dependence of scattering intensity (SI) of BSA with concentration. In Figures 1–6, the y-axis represents Rayleigh scattering intensity normalized with respect to incident light intensity. A.U. stands for arbitrary units.

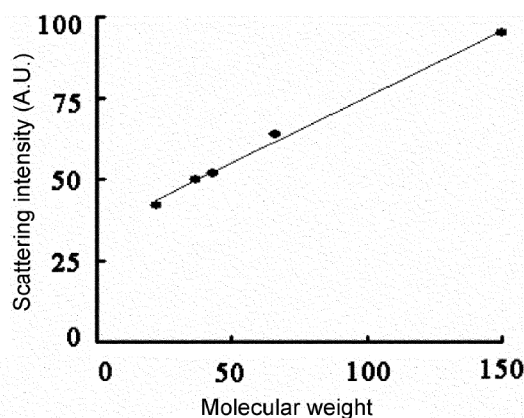


Figure 2. Dependence of scattering intensity on molecular weight. An equimolar concentration (45 μ M) of the following proteins in 50 mM Na-phosphate, pH 7.0, was used for scattering intensity measurement at 350 nm: chymotrypsinogen (25 kDa), β -lactoglobulin (36 kDa), ovalbumin (45 kDa), BSA (66 kDa) and yeast alcohol dehydrogenase (150 kDa). The linear dependence shows R^2 (regression coefficient) = 0.994. The extrapolated value on the y-axis indicates scattering intensity of the buffer and has not been subtracted from the sample readings.

constituting buffers usually remain within 500 Da and 5–10 mM respectively, background scattering intensity from buffers remains within limit. It is worthwhile to mention that the secondary scattering emissions arising from overtones and undertones of the excitation wavelength are also perceivable under the experimental conditions²⁹. These have not been considered here.

Dependence of scattering intensity

Scattering intensity from equimolar solutions of standard proteins of Mw between 14.7 and 150 kDa at 350 nm showed linear dependence on Mw and thus, also on the third power of Stoke's radius (Figure 2). This is on par with eqs (2) and (4). Scattering is an obligatory phenomenon as light travels from one medium to another due to change of refractive index (RI). When RI of a protein approaches that of the solvent, its scattering intensity becomes insignificant. To verify this, aqueous buffers were replaced by glycerol or sucrose. These compounds are inert, stabilize proteins, prevent denaturation and are highly viscous³⁰. Viscosity and RI of solvents are linearly proportional¹⁷. The decrease in scattering intensity from lysozyme, ovalbumin and BSA of fixed concentration (0.25 mg/ml) showed linear dependency as the composition of the aqueous buffer was gradually changed by 0–100% of glycerol. Scattering intensity was maximally reduced to 94 ± 4 , 92 ± 3 and $88 \pm 4\%$ respectively, for these three proteins. The dependency of BSA has been shown in Figure 3.

Scattering intensity from proteins is also affected by unfolding induced by 8 M urea or 6 M guanidinium.HCl. In this case, scattering intensity decreased due to penetration of solvent into the hierarchical structure of proteins, reducing the difference in RI between the solvent and the solute. High viscosity of the medium arising from high concentration of denaturants also reduces the scattering intensity. In contrast, an increase in scattering intensity is expected due to apparent expansion of protein molecules

due to unfolding. The resultant change in scattering intensity from lysozyme, ovalbumin and BSA after incubation with 8 M urea or 6 M guanidinium.HCl at equilibrium was not significant; usually within $\pm 10\%$. The Mw and the number of disulphide bridges of these proteins were 14, 45 and 67 kDa, and 3, 1 and 17 respectively. Relatively large number of disulphide bridges and/or strong hydrophobic interactions prevent unfolding of these proteins. However, under identical conditions of denaturation but in the presence of 10 mM of 2-ME that reduced the disulphide links, scattering intensity from these proteins was reduced by 84, 88 and 92% respectively. This indicates that maintenance of native conformation of proteins is a requirement for correct interpretation of the results of scattering.

During proteolysis, peptides of unknown number and undefined size are generated whereby the number of particles of decreasing size increases. These two opposing factors prevent prediction of change in scattering intensity during fragmentation of proteins, though a decrease is usually observed. Autodigestion of trypsin (50 $\mu\text{g/ml}$) in 50 mM Na-phosphate, pH 8.5 and at 37°C, as observed from a decrease in scattering intensity, followed a first-order kinetics having $k = 0.031 \pm 0.003 \text{ min}^{-1}$ for at least 25 min. Aforesaid results collectively justify the use of spectrofluorimeters in light scattering studies holding certain restrictions.

Subunit association–dissociation

During dissociation of a homomultimeric protein of the type $M_n \rightleftharpoons nM$, where M and n stand for subunit Mw and multimericity respectively, scattering intensity will be affected by two opposing factors; an increase due to the rise in the number of particles counteracted by a decrease due to reduction of their sizes. An analysis was done using eq. (5) on eight different proteins of native Mw 36–232 kDa and of multimericity 2 and 4. Mw and Stoke's radius (r_s) of the native multimeric molecules and dissociated subunits were adapted from the literature^{16,17}. Theoretical prediction of maximum difference of scattering intensity was $\pm 10\%$. Experimental data with these proteins after dissociation of the subunits indeed showed the same degree of variation (Table 1).

Strong interaction between trypsin and STI offers a special case where two non-identical proteins of equal Mw of 22 kDa interact in stoichiometric amount to form a stable homodimer-like complex. Effect of interaction of these molecules on scattering intensity was followed. As explained earlier, no significant change in scattering intensity was observed (Table 1).

Interacting proteins

In contrast to association–dissociation of homomultimers, interaction of a protein with another protein of unequal

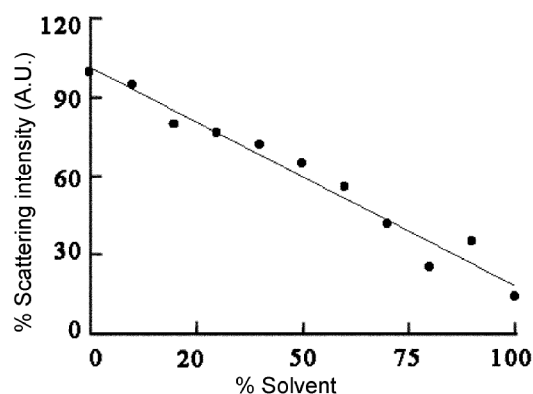


Figure 3. Linear dependency of scattering intensity of BSA (0.25 mg/ml) in 20 mM Na-phosphate, pH 7.5, at 350 nm containing variable percentage of glycerol as solvent. $R^2 = 0.965$.

Table 1. Change in scattering intensity with association–dissociation of homomultimeric proteins

Protein	Reaction (Mw (kDa), Stoke's radius (Å))	Scattering intensity (A.U. $\times 10^3$)			% Change predicted (observed)
		Initial	→	Final	
β -Lactoglobulin	$M_2 \rightarrow 2M$ (35, 26.47) (17.50, 20.50)	18.54	→	17.23	−7.06 (−2.50)
Epimerase (<i>Escherichia coli</i>)	$M_2 \rightarrow 2M$ (79, 35.75) (39.50, 27.80)	45.69	→	42.97	−5.95 (−7.30)
Alcohol dehydrogenase	$M_4 \rightarrow 4M$ (80, 35.90) (20.00, 22.60)	46.29	→	46.17	−0.21 (+2.50)
Hexokinase	$M_2 \rightarrow 2M$ (100, 38.99) (50.00, 30.00)	59.30	→	54.00	−8.93 (−2.30)
Glucose-6-phosphate dehydrogenase	$M_2 \rightarrow 2M$ (104, 38.99) (55.00, 30.20)	59.30	→	55.09	−7.09 (−2.76)
Epimerase (<i>Kluyveromyces fragilis</i>)	$M_2 \rightarrow 2M$ (150, 45.29) (75.00, 35.20)	92.90	→	87.22	−6.11 (−3.30)
Catalase	$M_4 \rightarrow 4M$ (232, 53.19) (58.00, 32.40)	150.48	→	136.0	−9.30 (−2.20)
Trypsin + STI ^a	$M_1M_2 \rightarrow M_1 + M_2$ (44, 28.70) (24.00, 22.90 + 20.00, 2.60)	23.64	→	23.55	−0.38 (+2.00)

^aTrypsin–STI complex is considered as a pseudo-homodimer because of similar molecular weights of the protein molecules.

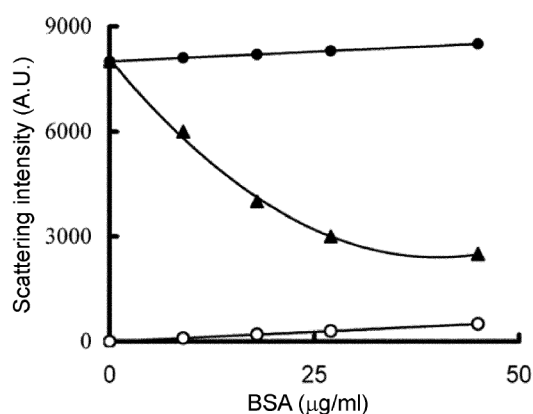


Figure 4. Dependence of scattering intensity of histone (45 μ g/ml) (\blacktriangle – \blacktriangle) in the presence of increasing concentration of BSA as indicated. Addition of BSA above 50 μ g/ml could not reduce scattering intensity of histone to any significant extent. Scattering intensity from BSA at the corresponding concentrations is represented by (\circ – \circ). Additive scattering intensity from BSA and histone as free entities in solution has been indicated by (\bullet – \bullet).

size could be followed with confidence. Binding between weight proportion of BSA (an acidic protein) and histone (a basic protein) could be clearly assessed as scattering from histone is gradually reduced in the presence of increasing concentration of BSA (Figure 4). It is noteworthy that histone–BSA interaction is not site-specific. Histone being composed of several discrete subunits, its molar concentration remains uncertain. Thus the binding is better conceived on a weight/weight rather than mole/mole ratio. The result indicated that for 45 μ g of histone, approximately 50 μ g of BSA was required to induce maximum fall in scattering intensity. This corresponds to saturation of binding at nearly 1 : 1 wt/wt ratio. Interac-

tion between a proteolytic enzyme (trypsin, 22 kDa) and a peptide (fibronectin type III-like peptide, 7.2 kDa) acting as an inhibitor was also successfully monitored, where a significant fall in scattering intensity was observed. These interactions were independently verified using FRET (fluorescence resonance energy transfer) analysis and biological assay³¹. Similarly, interaction between adenosine kinase (38 kDa) and cyclosporin (17 kDa) from *Leishmania donovani* was supported from alteration of scattering intensity¹¹.

Non-specific interactions

Equation (2) states that the scattering intensity contributed by each component of a heterogeneous system would be additive provided the components are non-interacting and sufficiently dilute to avoid intermolecular interactions (ideal solution). Linear dependence of scattering intensity with concentration was observed using several homogeneous proteins, e.g. lysozyme, ovalbumin and BSA at a relatively low range of concentration of 0–80 μ g/ml (Figure 5). Thereafter scattering intensity of three sets of the same proteins, e.g. lysozyme–ovalbumin, ovalbumin–BSA and BSA–lysozyme between the same concentration ranges was followed. Expected and observed scattering intensities were similar, but not exactly identical. A finite difference of 2–5% was observed (Figure 5). An important corollary from this result is that any deviation from this additive profile is likely to indicate an interacting system (non-ideal solution).

When the concentrations of the same proteins of the same sets were raised, deviation between expected (additive) and observed scattering became wider; e.g. in the

case of BSA-lysozyme and ovalbumin-lysozyme sets at pH 7.0, the difference was 6.5–7.0 fold (Figure 6). These reflect intermolecular interaction, ionic or otherwise, that

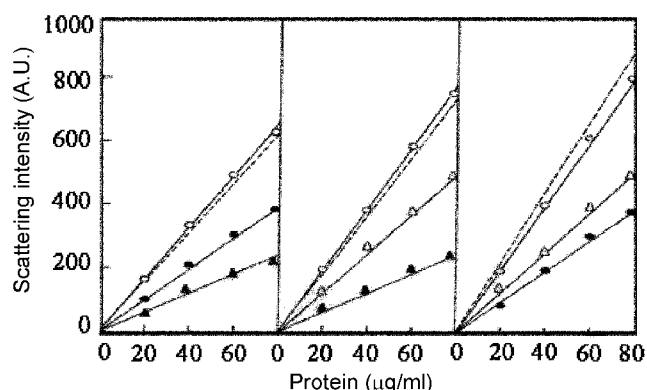


Figure 5. Profiles of scattering intensity of non-interacting proteins at low concentration. (Left) BSA-lysozyme and ovalbumin; (middle) ovalbumin and lysozyme, and (right) lysozyme and BSA. The following notations have been used; lysozyme (Δ), ovalbumin (\blacktriangle), BSA (\bullet). Scattering intensity observed in the presence of two proteins in each set has been presented by (\circ). The dotted line in each set was constructed using additive values of scattering intensity from individual proteins of each set.

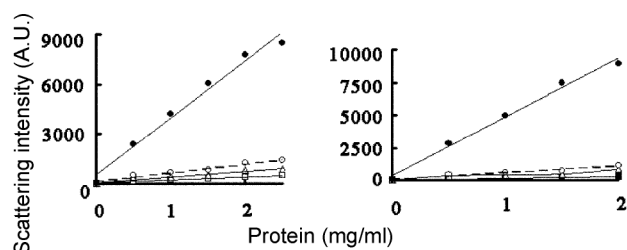


Figure 6. Scattering intensity of apparently non-interacting proteins at high concentration. (Left) BSA-lysozyme and (right) Ovalbumin-lysozyme where (Δ), (\blacksquare) and (\square) represent individual scattering intensity from lysozyme, ovalbumin and BSA respectively. Experimentally observed and additive scattering intensity for the two sets have been presented by (\bullet) and (\circ) respectively.

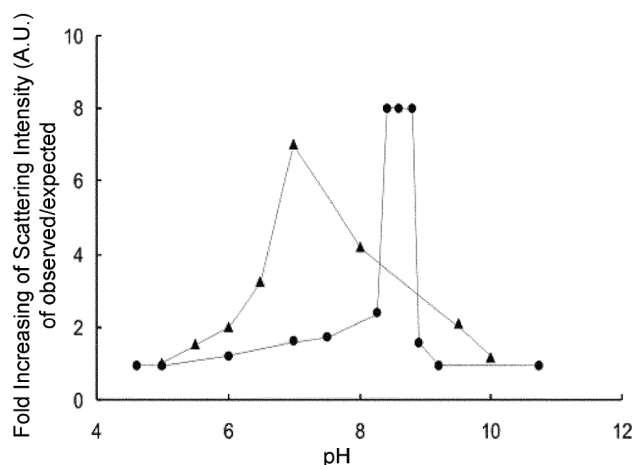


Figure 7. Dependence of fold increase of scattering intensity (observed/expected) on pH for BSA-lysozyme (\blacktriangle - \blacktriangle) and lysozyme-ovalbumin (\bullet - \bullet) solutions respectively. Protein concentration was 1.0 mg/ml each. The leveling-off at 8 on the y-axis scale indicates that scattering intensity is out-of-scale.

possibly affects the second virial coefficient of eq. (1), leading to such rise in scattering intensity. Since BSA (isoelectric point, $pI = 4.7$) and ovalbumin ($pI = 4.6$) are acidic and lysozyme ($pI = 10.7$) is basic, ionic interaction between the acidic and basic proteins appears to play a role at pH 7.0.

To minimize ionic interactions, the pH of the incubating buffer was varied between 4.0 and 11.0, expecting that at pI of one of the proteins, one interacting component will be neutral and the interactions will be minimum. The assumption was found to be true as the enhanced scattering intensity of the BSA-lysozyme set was reduced to normalcy at pH 4.7 or 10.7. Also, the enhancement of the scattering was maximum at pH 7.2, a point almost equidistant from the two pI s. The profile for ovalbumin ($pI = 4.6$) and lysozyme ($pI = 10.7$) followed the same pattern having maximum scattering in the middle of pH 4.6–10.73 (Figure 7). It will be of interest to see whether this protocol is suitable for the determination of pI of an unknown protein after its interaction with a reference protein. Change in solution character of a single component system can also be perceived after varying its concentration; for example, BSA shows three types of linear dependencies of scattering intensity with concentration in the range 0–25, 25–100 and 100–200 $\mu\text{g/ml}$ at pH 7.5 under identical experimental conditions³². Interestingly, all these results have been obtained using spectrofluorimeters.

Conclusion

In the absence of a dedicated instrument for measuring static and dynamic light scattering intensity from proteins in solutions, a spectrofluorimeter can be used to measure Rayleigh scattering intensity at 350 nm to monitor protein-protein interactions. In the presence of an aqueous buffer, a modified form of the Debye-Zimm relation holds good that is supported by experimental results. When the observed scattering intensity from two proteins in solution differs considerably from their combined individual contributions, non-ideal behaviour of the solution becomes prominent, indicating protein-protein interactions. The analysis can be extended to solution thermodynamics, including identification of protein multimers³³. However, this technique is not suitable to detect association-dissociation of homomultimeric proteins.

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