Rayleigh Light-Scattering Spectroscopy: Application to the Determination of Proteins Using Bromopyrogallol Red

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A method for the quantitation of proteins in aqueous solution involving the binding of bromopyrogallol red to proteins under acidic conditions has been developed. The binding of the dye to proteins is accompanied by an enhancement of Rayleigh light scattering at 332 nm; the scattering intensity is linear over the range 0.136—6.80 μg ml^{-1}. The reaction is completed immediately after mixing dye and protein solutions, and the scattering signal is stable for at least 3 h. There are very few interference with the method, most of which can be minimized by dilution.

Many techniques have been used to determine proteins, such as staining methods,[1,2] fluorometric methods,[3,4] spectrophotometric methods,[5,6] and chemiluminescent methods.[7,8] In this paper, a new method using Rayleigh light scattering in the determination of proteins is proposed.

Rayleigh light scattering (RLS) is an effective technique for studying the aggregation of dyes and porphyrins,[9—11] as well as the structures of various complexes and macromolecules.[12,13] Pasternack et al.[9] showed that porphyrin aggregating on a DNA template exhibits very strong scattering at wavelengths near to the absorption-band maximum of the aggregate. In this contribution, Huang first used RLS for analytical purpose to determine the quantities of nucleic acids with tetrakis(4-trimethylammoniumphenyl) porphyrin.[14] Because all of the absorption process are inherently associated with light scattering, all kinds of dyes have their own RLC spectra. It is possible to enhance the weak Rayleigh light scattering of small dye molecules through their binding with macromolecules, such as nucleic acids and proteins. It is found that the light scattering of bromopyrogallol red (BPR) is greatly enhanced by proteins, based on this, a new protein assay method is put forward.

Basic theories for enhanced light scattering has been described previously.[9,15,16] The intensities of the Rayleigh light scattering is proportional to the volume of the species and $C\lambda^{-4}$ $[(dn/dC)^2 + (dk/dC)^2]$, where λ is the wavelength, C is the molarity of the solution, dn/dC and dk/dC are, respectively, the real and imaginary parts of the scattering's polarizability, and dn/dC and dk/dC are wavelength-dependent terms. When the wavelength and experimental conditions are fixed, the scattering intensity is only proportional to the molar concentration of the solute. This is the quantitative basis for the following assay.

The assay described herein is characterized by both sensitivity and simplicity. Since the light-scattering measurements can be easily performed on a fluorimeter, this assay can be run in many laboratories.

Experimental

Apparatus. The RLS intensity and spectra were obtained with a Shimadzu model 540 fluorimeter (Japan). Cells of 1-cm path length were used for detection. The absorption spectra were obtained with a Shimadzu Model 265 recording spectrophotometer (Japan). The pH values were measured with a model 821 pH meter (Zhong Shan University, P. R. China).

Reagents. All of the chemicals were of analytical reagent or the best grade commercially available. All stock solutions of the proteins and chemicals were prepared in doubly deionized water. BPR was purchased from Shanghai Chemical Plant (China), and used as supplied. The BPR reagent (0.1 %) was prepared in ethanol (20%). The buffer solutions used were sodium citrate–citric acid (pH 3.96). Several proteins (trypsin, protamin sulfate, hemoglobin (bovine), lysozyme, bovine serum albumin (BSA), human serum albumin (HSA), human IgG and egg albumin) were obtained from Sigma. α-Chymotrypsin and pepsin were obtained from Shang Hai Biochemistry Institute (China). The protein concentrations were determined spectrophotometrically at 280 nm using the following $\varepsilon_{280}^{1\%}$ values: BSA, 6.6;[17,18] lysozyme, 26.04;[18] HSA, 5.3;[17,19] human IgG, 13.8;[18] egg albumin, 7.5.[18] The concentrations of hemoglobin, trypsin, protamin sulfate, pepsin and α-chymotrypsin were determined as follows;[20] protein concentration (μg ml^{-1}) = $144 \times (A_{215} - A_{225})$. A_{215} and A_{225} are the absorbances at 215 and 225 nm measured using a 1-cm cell.

Procedures. In most of the experiments, 0.5 ml of the BPR reagent (0.1%), 2 ml of a buffer (pH 3.96), and 0.5—2.0 ml of a 0.034 mg ml^{-1} BSA standard solution or protein samples were mixed and diluted to 10 ml with water, which were held in 1-cm quartz cuvettes. The Rayleigh light-scattering spectra were obtained with the excitation and emission monochromators of the fluorimeter scanned synchronously through a range covering 300—700 nm, The RLS intensity was obtained with the excitation and emission wavelengths set at 332 nm. The standard curve, obtained by plotting the standard protein concentrations versus the corresponding scattering intensity, was used to determine the protein concentration in unknown samples. The standard and unknown samples were prepared and run simultaneously under the same assay conditions.

Results and Discussion

Reaction and Spectra. The reaction between BPR and proteins occurs rapidly at room temperature (< 2 min). The scattering intensity is stable for at least 3 h. Thus, this assay doesn't require any crucial timing. Two kinds of mixing sequences were investigated. One was to mix BPR and the buffer first, after which BSA solutions were added. In the other the buffer solution was added to mixture of BSA and BPR. The results have shown that the addition sequence of reagents greatly affected RLS (data not shown). Mixing the buffer and BPR first results in a higher sensitivity than the other way.

RLS and the absorption spectra are shown in Fig. 1. The addition of BSA greatly enhances the scattering intensity of BPR at 332 nm, though it almost doesn't affect the absorbance of BPR at this same concentration of BSA. Weak scattering is observed in the 540—560 nm range. This is consistent with the high absorptivity in this range (see Fig. 1a).

pH Effect. The effect of the pH on RLS was investigated for pH 2.6—5.0. Sodium citrate–citric acid was used to control the pH. The scattering intensity of the assay system is greatly affected by the pH, whereas the RLS of the reagent blank is not affected. By affecting the nature and quantity of the electric charge on the proteins, the pH has an effect on the binding ability of the dye and proteins. Thus, the light-scattering signals change with the pH. As shown in Fig. 2, by plotting the scattering intensity against the pH, at pH 3.96 the scattering intensity reaches its maximum. Thus, pH 3.96 was chosen for the assay.

The effects of the pH on RLS of mixtures of BPR and other proteins were also investigated. The optimum pH values of the assays are given in Table 1. It can be seen that the pH is one of the important factors that result in the following discussed protein-to-protein variability.

Protein-to-Protein Variability. The individual responses of the various proteins assayed in the system are shown in Fig. 3. The linear range of BSA is 0.136—6.80 μg ml^{-1}. The scattering of points around the line drawn in the graph makes it difficult to determine the exact amount of protein present in a given sample. No obvious enhancement of RLS can be observed with pepsin, egg albumin and α-chymotrypsin. It was reported earlier[9,10] that the intensity of an enhanced RLS signal appears to depend sensitively on electronic properties of the individual chromophores, the strength of the electric interaction between the chromophores and proteins, and the size of the thus-formed complex. Since both the sizes of the protein molecules and the binding abilities between BPR and the proteins vary with different kinds of proteins, it can be concluded that a protein-to-protein

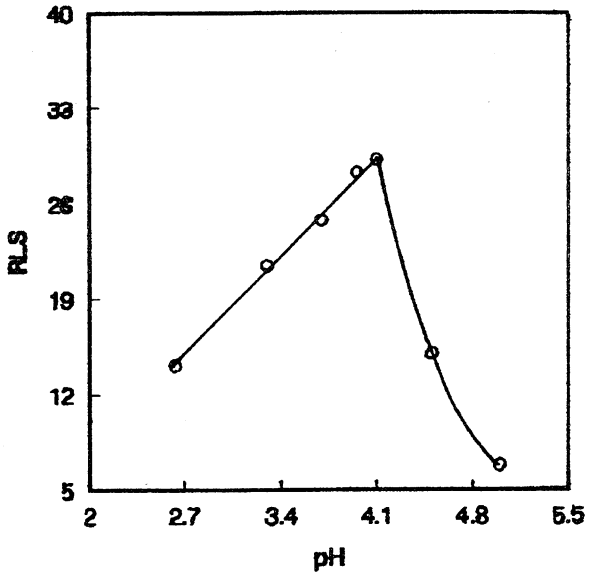

Fig. 2. Effect of pH on the BPR standard assay with concentrations of BPR and BSA 0.005% and 3.40 μg ml^{-1} (duplicate measurements.)

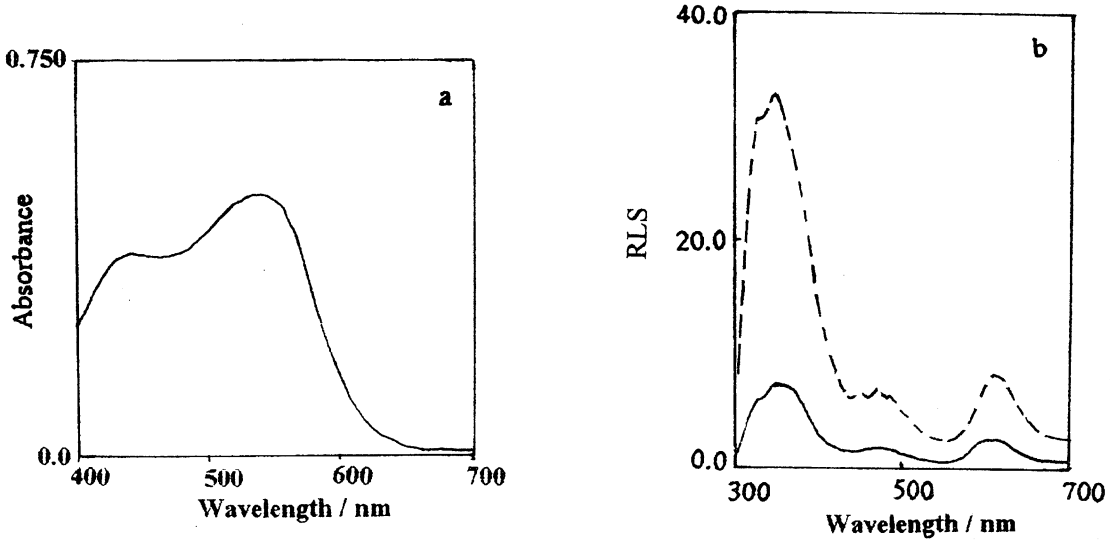

Fig. 1. Absorption and Rayleigh light scattering spectra at pH 3.96. (a) Absorption spectrum for BPR, (b) Rayleigh light scattering spectra for BPR (solid line) and mixture of BPR and BSA (dashed line).

Table 1. Optimum pH for Proteins Assay

Protein	Optimum pH
HSA	3.96
Trypsin	>5.0
Human γ-IgG	3.7–4.5
Egg albumin	3.7
Protamin sulfate	3.96–4.5
Lysozyme	3.7–4.5
Hemoglobin	4.1
α-Chymotrypsin	<2.6
Pepsin	NA[a]

a) No RLS enhancement of BPR was observed by addition of pepsin at pH range 2.6—5.0.

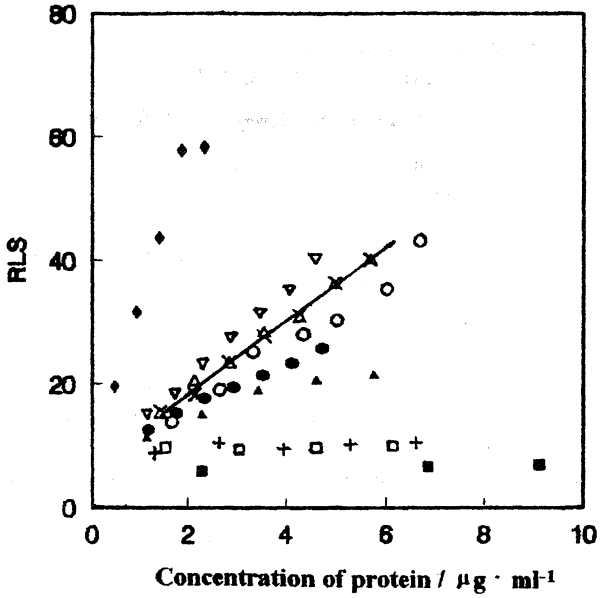

Fig. 3. RLS response pattern for various proteins. BSA (×), HSA (△), trypsin (○), hemoglobin (●), lysozyme (▽). protamin sulfate (◆), human γ-IgG (▲), egg albumin (+), pepsin (■), and α-chymotrypsin (□).

variability is difficult to avoid in this RLS technique.

Interfering Substances. In studies concerning the interferences of some substances with the BPR assay, BSA samples were premixed with interfering substances, then assayed at 3.40 μg ml^{-1} BSA. The final concentrations of substances are listed in Tables 2 and 3. It can be seen that few substances interfere with the BPR assay (Table 2). Salts, Fe(III) and Hg(II) have negative effects on the scattering intensity of the assay system, whereas ethanol and urea increase the RLS of BPR-BSA mixtures. All of these interferences can be minimized dilution. Moreover, this assay is not interfered with by amino acids (Table 3). Except for urea, all of the other substances listed in Tables 2 and 3 have no obvious effects on the scattering intensity of BPR. Thus, it may be by affecting the binding ability of BPR and BSA that some substances show interferences on the scattering intensity of the assay system.

The effects of detergents (hexadecyltrimethylammonium bromide (HTAB), β-cyclodextrin (β-CD), sodium dodecyl sulfate (SDS) and Triton X-100) on the assay were tested at a BSA concentration of 3.40 μg ml^{-1}. The detergents, such as Triton X-100, β-CD, and SDS, didn't affect the RLS of BPR (data not shown). The addition of HTAB prevented BPR from binding to BSA. The reaction between anions of BPR and the oppositely charged HTAB resulted in the same

Table 2. Effect of Interfering Substances

Sample (BSA)	Change in RLS	BPR assay (μg ml^{-1}BSA found)[a]
—	—	3.40
0.8mg ml^{-1}glucose	0.000	3.40
4.75% ethanol	0.288	4.38
19.0% ethanol	0.447	4.92
2.06 μg ml^{-1}sodium laurate	0.006	3.42
0.4 M urea	0.256	4.27
0.8 M urea	0.374	4.67
0.5 mM EDTA	−0.044	3.25
1.0 mM ascorbic acid	−0.024	3.32
50 mM NaCl	−0.009	3.37
0.35 M NaCl	−0.238	2.59
20 mM NH_4Cl	−0.018	3.34
50 mM NH_4Cl	−0.168	2.83
50 mM KCl	0.024	3.48
8.0 μMFe(III),(chloride)	−0.259	2.52
2.5 μM Pb(II), (nitrate)	0.000	3.40
4.0 μM Zn(II), (chloride)	−0.018	3.34
4.0 μM Ca(II), (chloride)	−0.026	3.31
4.0 μM Mg(II), (chloride)	−0.018	3.34
2.0 μM Mn(II), (nitrate)	−0.018	3.34
8.0 μM Cu(II), (nitrate)	−0.018	3.34
4.0 μM Al(III), (salfate)	−0.015	3.35
4.0 μM Cd(II), (chloride)	0.009	3.43
4.0 μM Cr(III), (chloride)	0.006	3.42
4.0 μM Hg(II), (nitrate)	−0.262	2.51
4.0 μM Ni(II), (nitrate)	−0.003	3.39
10.0 μM Co(II), (nitrate)	−0.018	3.34

a) Average value from at least two measurements (M=mol dm^{-3}).

Table 3. Effect of Amino Acids

Amino acid	Change in RLS	BPR assay (μg ml^{-1}BSA found)[a]
—	—	3.40
L-Pro	−0.015	3.35
L-Try	−0.059	3.20
L-Cys	−0.026	3.31
L-Asn	−0.038	3.27
L-Leu	−0.032	3.29
L-Glu	−0.056	3.21
Gly	0.006	3.42
L-Arg	−0.029	3.30
L-His	0.024	3.48
L-Lys	0.026	3.49
L-Phe	0.000	3.40
L-Ser	0.003	3.41
L-Tyr	−0.044	3.25
L-Ala	0.012	3.44

a) Average value from at least two measurements. The final concentrations of amino acids are all at 16.0 μg ml^{-1}.

responses of the reagent blank and the assay system (Fig. 4). β-CD had no great effect on the assay. Triton X-100 increased the RLS at first, then decreased it above a detergent concentration 0.03%. Negatively charged SDS neutralized the positive charge on a protein, which may reduce the binding number of BPR to the protein, resulting in a decrease in the scattering intensity.

Effect of the BPR Concentration. The Effect of the BPR concentration (0.0005—0.009%) on the scattering intensity was studied for the BSA (1.70—8.50 ml^{-1}) standard assay. The RLS yield is defined as the slope of the graph of the scattering intensity against the BSA concentration (RLS/μg protein ml^{-1}).

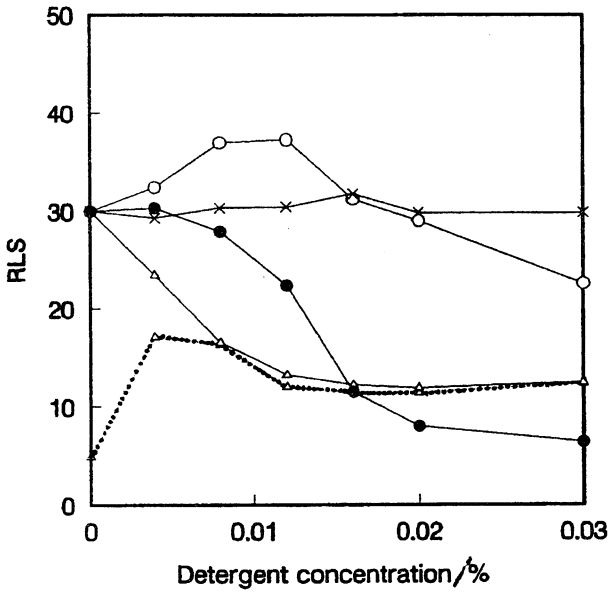

Fig. 4. Effect of detergents on the scattering intensity of 0.005% BPR (···), and mixture of 0.005% BPR and 3.40 μg ml^{-1} BSA (—). detergents: SDS (●), β-CD (×), Triton X-100 (○), and HTAB (△).

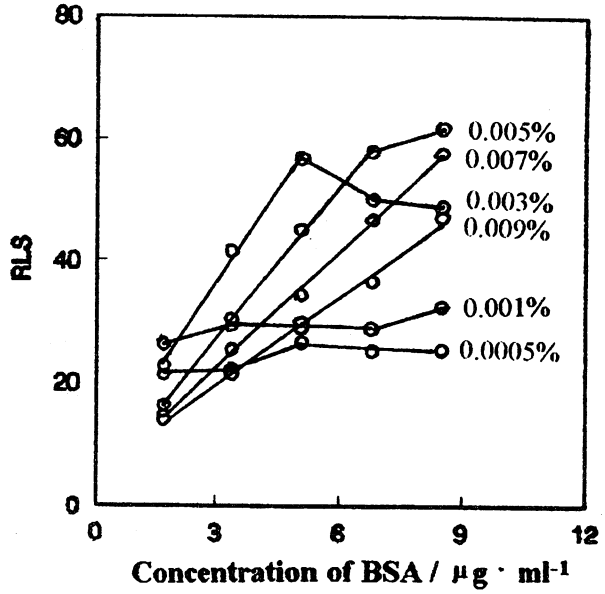

Fig. 5. Standard assay of BSA for a range of BPR concentrations (0.0005—0.009%).

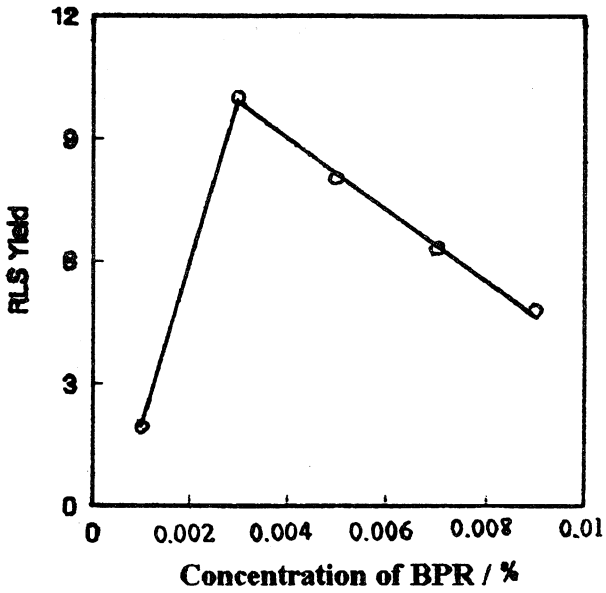

Fig. 6. RLS yields for a range of BPR concentrations (0.0005—0.009%).

The concentration of BPR affects the RLS of BPR-BSA mixture and the RLS yields. As shown in Fig. 5, along with an increase in the dye concentration, the linear range of the standard curve increases. This may indicate the saturation of binding between BPR and BSA at a lower concentration of BPR. It can be clearly seen from Fig. 6 that the maximum yield in this assay is reached at a BPR concentration of 0.003%. At a higher BPR concentration, the decrease in the RLS yields may be due to the higher concentration of free dye which results in a smaller enhancement of the scattering.[16] In order to obtain a wider linear range, the BPR concentration at 0.005% was chosen for all of the measurements.

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