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The high-sensitivity determination of protein concentrations by the enhancement of Rayleigh light scattering of Arsenazo-DBN

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A new Rayleigh light scattering (RLS) assay of protein is presented in this paper. At the optimum pH 4.10, the weak RLS of Arsenazo-DBN can be greatly enhanced by the addition of proteins due to the interaction between protein and Arsenazo-DBN. Based on this, the reactions of Arsenazo-DBN and proteins, including bovine serum albumin, human serum album, γ-globulin, egg albumin, lysozyme and trypsin, were studied. A new quantitative determination method for proteins has been developed. The linear range for human serum albumin, for example, is 0.085–34.62 μg mL^{-1} with a detection limit of 44.8 ng mL^{-1}. Besides high sensitivity, the method is characterized by good reproducibility, rapidity of reaction, good stability, and few interfering substances. The determination of the proteins in human serum and urine samples by this method give results very close to those obtained using Coomassie Brilliant Blue G-250 colorimetry, with relative standard deviations of 0.7–2.5%.

Introduction

The quantitative assay of protein is very important in biochemistry and clinical medicine. To date, widely used assays for proteins include the Lowry,[1] Coomassie Brilliant Blue (CBB),[2,3] Bromophenol Blue[4] and Bromocresol Green[5] methods. However, these methods have their disadvantages in terms of sensitivity, selectivity, stability and simplicity. For example, the Lowry, the Bromophenol Blue and Bromocresol Green methods have relatively poor sensitivity and are seriously interfered by substances often found in biological samples. These limitations are now partially overcome by some new methods, such as chemiluminescent[6] and fluorometric[7] analysis. It is important for biochemists to develop some improved methods for protein assay. Resonance light-scattering (RLS) is a phenomenon of elastic light-scattering. The light scattering intensity at some wavelengths becomes much higher than that of usual light scattering and can be measured with a common spectrofluorometer.[8] Recently it has been used in analytical chemistry by Tong and co-workers,[9,10] 3-[(2-Arsonophenyl)azo]-6-[(2,6-dibromo-4-nitrophenyl)azo]-4,5-dihydroxy-2,7-naphthalenedisulfonic acid (Arsenazo-DBN) is a good chromogenic reagent (its structure is shown in Fig. 1), but except for use in the determination of some rare earth metals,[11] it has not yet been paid much attention. In this report, a novel method for determination of protein is presented. It is based on the binding reaction of protein with this spectroscopic probe, Arsenazo-DBN. The weak RLS of Arsenazo-DBN can be enhanced greatly by the addition of proteins. The application of Arsenazo-DBN as a probe for proteins leads to a particularly stable, simple and selective system, permitting a limit of detection of 44.8 ng mL^{-1} for human serum albumin (HSA). This method is much more sensitive than most of the accepted and reported dye- and complex-binding methods, and it is suitable for routine application.

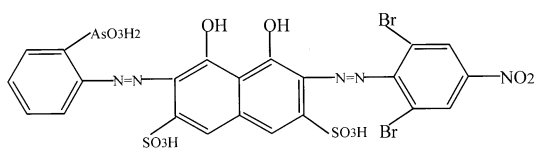

Fig. 1 The structure of Arsenazo-DBN.

Experimental

Instruments

The spectrum and the intensity of Rayleigh light scattering were obtained with a Hitachi M-850 fluorescence spectrometer (Tokyo, Japan) with an 150 W xenon lamp and a 1 cm quartz cell. All of the spectral data were given with correction. The excitation and emission band widths were 5 nm. The absorption spectra in the visible region were recorded with a 721 spectrophotometer (Shanghai Analytical Instrument Factory, China). The pH measurements were made with a model pHS-2 pH meter (Shanghai Analytical Instrument Factory, China).

Reagents

Arsenazo-DBN was obtained from Fanghua Xueyuan Shiyan Huagongchang (China) and was directly dissolved in water to prepare a stock solution of 1000 μmol L^{-1} and diluted to 250 μmol L^{-1} with water as working solution just prior to use. Tween-20, sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), and Coomassie Brilliant Blue G-250 (CBB G-250) were obtained from Fluka (Buchs, Switzerland). The working solution (1.17×10^{-5} mol L^{-1}) for CBB G-250 was prepared by dissolving 0.1 g of the crystals in 50 ml of 95% ethanol, and then mixing with 100 mL 85% phosphoric acid. The mixture was then diluted to 1000 mL with doubly-distilled water. The Britton–Robinson buffer (pH 4.10, and composed of 100 mL of the acid mixture of 0.04 mol L^{-1} H_3PO_4, 0.04 mol L^{-1} CH_3COOH, 0.04 mol L^{-1} H_3BO_3 and 25 mL of 0.2 mol L^{-1} NaOH) was used to control the acidity of tested solutions.

Bovine serum albumin (BSA), human serum albumin (HSA), γ-globulin (γ-IgG), egg albumin (Alb), lysozyme (Lys) and trypsin (Try) (Sigma, St Louis, MO, USA) were directly dissolved in water to prepare stock solutions of 250 μg mL^{-1}, and stored at 0–4 °C. The working solutions were obtained by diluting the stock solutions with water just prior to use. The precise concentrations were determined spectrophotometrically at 280 nm with the $\varepsilon^{1\%}$ (the absorbance of 1% m/v solution with a 1 cm cell, cm^{-1} ml^{-1} g) values for: BSA, 6.6; Lys 26.04; HSA 5.3; γ-IgG 13.8; Alb 7.5. The Try concentration (μg mL^{-1}) =

144($A_{215} - A_{225}$), where A_{215} and A_{225} were absorbance at 215 nm and 225 nm measured with a 1 cm cell.

All other chemicals were of analytical grade or the best grade commercially available. Human serum and urine samples were provided by the Hospital of Lanzhou University. The serum samples were diluted 3000-fold with doubly deionized water and the urine samples were directly used for determination.

General procedures

Britton–Robinson buffer (1 mL), an appropriate quantity of the working solution of Arsenazo-DBN and of protein (or sample) were mixed, then diluted to 10.0 mL with water and stirred thoroughly. The RLS spectra were obtained with the excitation and emission monochromators of the fluorometer scanned synchronously (0.0 nm interval between excitation and emission wavelength) through the wavelength range of 300–600 nm. All measurements were obtained against the blank treated in the same way without proteins. Based on these spectra, the intensity of RLS was measured with the excitation and emission wavelengths at 420.0 nm.

Results and discussion

Reaction and spectral characteristics

The reactions between Arsenazo-DBN and proteins at room temperature occur within 2 min. The scattering intensity is stable for at least 3 h and is not affected by the addition sequence. The spectra of Arsenazo-DBN and Arsenazo-DBN–HSA at pH 4.10 are shown in Fig. 2. The Arsenazo-DBN absorption spectrum changes slightly when HSA is added, while the RLS spectra changes a lot, which indicates interaction between Arsenazo-DBN and proteins. Here it can be seen that the peak of light scattering of the complex does not appear within the envelope of its absorption spectrum, so the reaction system could be treated as a transparent solution. This may be because the molecule simultaneously absorbs the incident light and the scattered light, resulting in its weak light scattering intensity in the absorption region. Therefore, the quantitative basis for this assay is in accordance with the Rayleigh formula:[12]

$$R_\theta = 9\pi^2 N_0 v^2 (1 + \cos^2\theta)[(n_1^2 - n_2^2)/(n_1^2 + 2n_2^2)]^2/2\lambda^4$$

where R_θ is the Rayleigh ratio at 90° scattering angle (the Rayleigh ratio describes the scattering ability of the system), n_1 and n_2 are the refractive index of the solute and medium respectively, λ is the wavelength, v is the size of the scattering particles, and N_0 is the number of particles per unit. When the scattering species of the system is fixed, then v is constant. Because the scattering particle is an electrostatic Arsenazo-DBN–protein complex, the number of scattering particles is determined by the concentration of protein added at a fixed concentration of Arsenazo-DBN. Therefore N_0 is proportional to the concentration of the protein added, *i.e.*, under other experimental conditions that are fixed, the scattering light intensity I is determined only by the protein concentration C ($I = kC$).

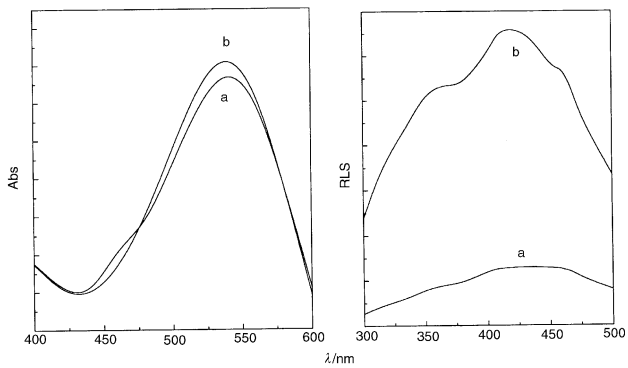

Fig. 2 Spectra at pH 4.10. Absorption spectrum (left) and RLS spectrum for Arsenazo-DBN (a) and the complex of Arsenazo-DBN and HSA (b). Conditions: HSA, 10 μg mL^{-1}, Arsenazo-DBN 25.0 μmol L^{-1}, pH 4.10. Spectrofluorometer parameter: excitation and emission slits width, 5 nm; PM gain, low; scan speed, 60.

Effect of solution acidity

Fig. 3 shows that pH significantly affects the RLS intensity, due to the dissociation and ion charge of Arsenazo-DBN and proteins. The optimum pH conditions are found to be 3.78–4.35 for HSA, BSA, Alb, and 4.10–7.00 for Lys and γ-IgG. Due to the pH sensitivity of the RLS measurement, the protein solution should be always be buffered, preferably at pH < 4.5. In order to simplify the method and compare results of all proteins, pH 4.10 Britton–Robinson buffer is recommended for all experiments.

Effect of Arsenazo-DBN concentration (C_{DBN})

The scattering intensities of solutions containing various concentrations of Arsenazo-DBN and HSA were measured. It was found that the C_{DBN} affects the RLS sensitivity. When the C_{DBN} was low, the linear range (*Lr*) was narrow, which may imply saturation of Arsenazo-DBN binding to protein. When the C_{DBN} was high, the slopes of the standard curves (corresponding to the sensitivity, *S*) decrease due to a lower enhancement of the scattering. In order to get a higher sensitivity (*S*) and a wider linear range (*Lr*), the C_{DBN} must be carefully chosen. Here we define a new criterion (*Cr*):

$$Cr = S \times Lr$$

The relationship between *Cr* and C_{DBN} is obtained with nonlinear regression analysis:

$$Cr = 13.78 + 8.27\exp^{\frac{(C_{DBN}-24.81)^2}{127.04}}$$

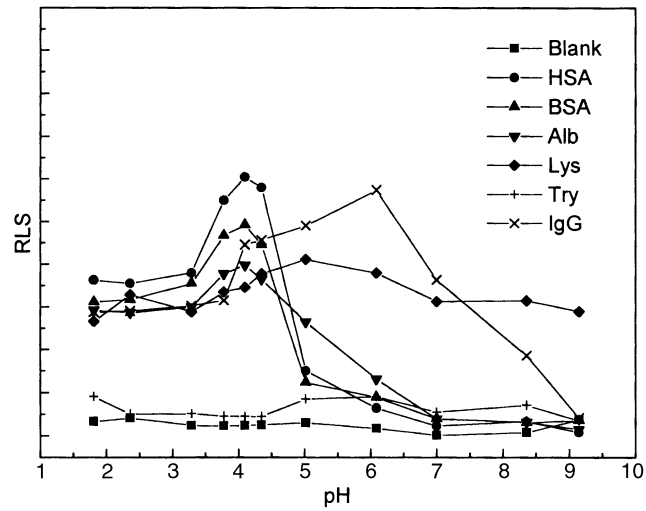

Fig. 3 The dependence of RLS on pH. Concentration: Arsenazo-DBN, 25 μmol L^{-1}; protein, 10 μg mL^{-1}, λ = 420.0 nm.

It can be seen that *Cr* reaches the maximun value when C_{DBN} = 24.81 μmol L^{-1}. In this paper, 25.0 μmol L^{-1} Arsenazo-DBN was used.

Effects of surfactants

From Fig. 4, it can be seen that surfactants affect the intensity of RLS seriously. The RLS of Arsenazo-DBN and the complex increases with the concentration of SDS and of CTAB, but these substances have a negative effect on the RLS when their concentrations are above 0.003% and 0.004% separately. This may be because, *e.g.*, the negatively charged SDS neutralizes the positive charge on the proteins so as to reduce the binding of Arsenazo-DBN to proteins, resulting in a decrease of scattering intensity. The reaction between Arsenazo-DBN anion and oppositely charged CTAB results in the same effects on the reagent blank and the assay system. Tween-20 has no significant effects on Arsenazo-DBN and the complex.

Interfering substances

As shown in Tables 1 and 2, amino acids interfere very little, and few other ions tested interfere with this assay, so no special preparation was taken before sample determination.

Calibration graphs

The results of application of the method for several proteins, such as BSA, HSA, γ-IgG, Alb and Lys is presented as standard regression equations in Table 3. Different proteins have different isoelectric points. At the same time, the weight, size and shape of the molecules are also different, so the RLS signals for various proteins are different. The limit of detection was calculated using the following formula:[13]

$$C_L = kS_{b1}/S$$

where C_L is the limit of detection; k is a constant related to the confidence level, using the suggestion of the IUPAC, $k = 3$; S_{b1} is the standard deviation of eight blank measurements and S is the slope of the calibration graph.

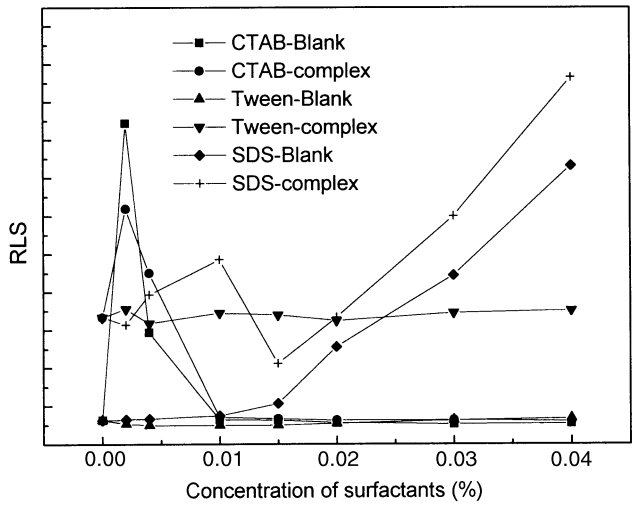

Fig. 4 Effect of surfactants on the RLS intensity. HSA: 10 μg mL^{-1}, Arsenazo-DBN 25.0 μmol L^{-1}, pH 4.10.

Measurement of sample

The present method was applied to the determination of total proteins in human serum and urine samples. Table 4 shows the results, which are very close to those obtained using the CBB method. Therefore the determination of protein by this method is reliable, practical, sensitive and simple.

Conclusion

The method described here is based on the interaction of protein and Arsenazo-DBN, and utilizes the resonance light-scattering

Table 1 Effects of amino acids on RLS[a]

Amino acid (40.0 μg mL^{-1})	Change of RLS intensity (%)	Amino acid (40.0 μg mL^{-1})	Change of RLS intensity (%)
L-Asp	−0.21	L-Glu	−0.35
L-Leu	−0.31	L-Cys	−4.32
L-Leu	4.65	L-Arg	−6.40
L-Phe	0.56	L-Lys	−1.06
L-Pro	0.56	L-Ser	2.13
L-Try	−3.07	L-Thr	−4.65
L-His	−6.21	L-Gly	2.14

[a] HSA, 10.0 μg mL^{-1}; Arsenazo-DBN, 25.0 μmol L^{-1}; pH 4.10, average of five measurements.

Table 2 Effect of interfering substances on RLS[a]

Interfering substances (20.0 μg mL^{-1})	Change of RLS intensity (%)	Interfering substances (20.0 μg mL^{-1})	Change of RLS intensity (%)
Al^{3+}, chloride	11.21	Mn^{2+}, chloride	−2.32
Ca^{2+}, chloride	1.03	Na^{+}, chloride	0.09
Cd^{2+}, chloride	11.13	Sn^{2+}, chloride	1.45
Co^{2+}, chloride	0.05	Ethanol, 5%	4.50
Cr^{3+}, nitrate	−3.74	Methanol, 5%	3.12
Cu^{2+}, chloride	−2.01	Urea, 1.0 mol L^{-1}	0.52
Fe^{3+}, chloride	5.32	Glucose, 1.0 mol L^{-1}	2.13
Hg^{2+}, chloride	3.52	$H_2PO_4^-$, K^+, 1.0 mol L^{-1}	0.31
K^{+}, chloride	0.02	HCO_3^-, Na^+, 1.0 mol L^{-1}	0.12
Mg^{2+}, chloride	−3.12	Guanidine, Cl^-, 1.0 mol L^{-1}	0.43

[a] HSA 10.0 μg mL^{-1}, Arsenazo-DBN 25.0 μmol L^{-1}, pH 4.10, average of five measurements.

Table 3 Standard regression equations of various proteins[a]

Protein	Standard regression equation (C, μg mL^{-1})	Regression coefficient	Linear range/μg mL^{-1}	Determination limits (D_L)/ng mL^{-1}
BSA	$Y = -0.886 + 0.445\ C$	0.999	0.105 − 33.00	67.4
HSA	$Y = -1.46 + 0.669\ C$	0.995	0.085 − 34.62	44.8
Alb	$Y = -0.49 + 0.415\ C$	0.994	0.25 − 30.00	72.3
Lys	$Y = -0.75 + 0.411\ C$	0.991	0.13 − 30.10	73.0
γ-IgG	$Y = -0.10 + 0.34\ C$	0.998	0.11 − 28.72	88.2
Try	RLS too low			

[a] Arsenazo-DBN 25.0 μmol L^{-1}, pH 4.10, average of five measurements.

technique. The mechanism of the enhancement of RLS by proteins is still under study, but this novel spectrophotometry can undoubtedly be used in protein determination. Compared with other general methods, the main advantages of the enhanced RLS technique are its high sensitivity, reproducibility, and it is unaffected by amino acids and most of the common ions. It has been used in an ordinary biochemical laboratory and the results are reliable.

Table 4 Determination results for human plasma and urine samples[a]

	Protein[b]			
Samples (No.)	This method (n = 6)	RSD (%)	CBB method (n = 6)	RSD (%)
Serum 1#	61.1	2.5	60.0	3.0
Serum 2#	64.1	1.2	70.2	1.6
Serum 3#	87.2	1.2	89.2	2.3
Serum 4#	94.3	1.3	102.3	4.7
Serum 5#	100.1	0.7	94.5	3.1
Serum 6#	84.2	0.87	87.6	2.6
Urine 1#	11.2	0.97	10.7	1.2
Urine 2#	8.6	1.3	9.1	1.3
Urine 3#	28.7	2.3	29.4	1.5

[a] Obtained from the Hospital of Lanzhou University. [b] Protein concentration in serum is expressed in mg mL^{-1} and that in urine is in mg L^{-1}.

Acknowledgements

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