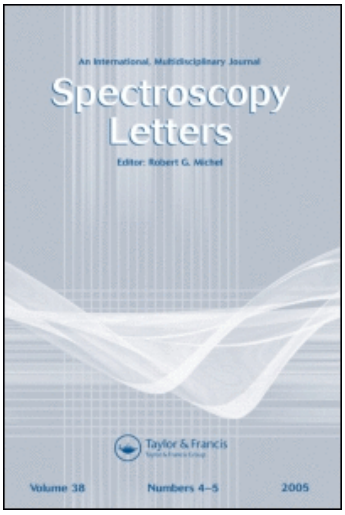

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RAYLEIGH LIGHT SCATTERING ENHANCING REACTION OF DIBROMOCHLOROARSENAZO AND PROTEINS SYSTEM AND ITS ANALYTICAL APPLICATION

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ABSTRACT

Based on the enhancement effects of Rayleigh light scattering (RLS) on Arsenazo-DBC, a novel quantitative method for the determination of proteins in aqueous solutions has been proposed. The reaction of Dibromochloroarsenazo (Arsenazo-DBC) and five proteins (BSA, HSA, egg albumin, human γ-IgG, Lysozyme) has been studied. Under optimal conditions the dynamic ranges for proteins were 2.5–60.0 $\mu g \cdot ml^{-1}$, and the detection limits for HSA and BSA were at 98.50 $ng \cdot ml^{-1}$ and 88.10 $ng \cdot ml^{-1}$, respectively. Comparing with other methods, the method is simple, practical and relatively free from interference from coexisting substances. The method was employed for the determination of total protein in human serum with satisfactory results.

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Key Words: Rayleigh light scattering; Dibromochloro-arsenazo; Protein

INTRODUCTION

The determination of proteins is a basic requisite in clinical test and laboratory practice. The quantitative analysis of proteins at trace levels is currently of considerable importance and interest, since protein analysis is vital to many areas of analytical biochemistry and biotechnology. Since proteins show, by their very nature, poor UV absorption and small levels of fluorescence, other methods of lowering the detection limits are important. To date, the widely used assays for proteins are the Lowry,[1] Bradford,[2–4] Bromophenol Blue[5] and Bromocresol Green[6] methods. However, these methods have their disadvantages. The limitations are now partially overcome by some new methods, such as chemiluminescent[7] and fluorometric[8] analysis. It is important for biochemists to develop some improved methods for protein assay.

The scattering light is well applicable to polymer science[9–10]. The technique also has great potential in trace analysis because of its sensitivity. Recently, the resonance light scattering technique has been used in analytical chemistry[11–13]. Arsenazo-DBC (its structure shown in Figure 1) is a good chromogenic reagent that, except for being used in the determination of some rare earth metals[14], has not yet received much attention. In this report, a novel method for determination of protein has been developed. It is based on the binding reaction of protein with this spectroscopic probe, Arsenazo-DBC. The weak RLS of Arsenazo-DBC can be enhanced greatly by the addition of proteins. The application of Arsenazo-DBC as a probe for proteins leads to a particularly stable, simple and selective system, permitting a limit of detection of 88.10 $ng \cdot ml^{-1}$ for bovine serum albumin (BSA). This method is much more sensitive than most of the accepted and

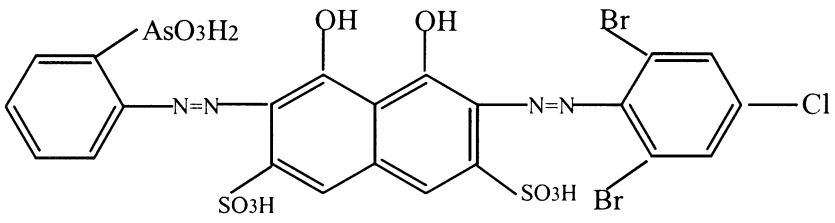

Figure 1. The structure of Arsenazo-DBC.

reported dye- and complex-binding methods, and it is suitable for routine analysis applications.

EXPERIMENTAL SECTION

Instrumentation

The spectrum and the intensity of Rayleigh light scattering were obtained using a HITACHI M-850 fluorescence spectrometer (Tokyo, Japan) with a 150-W Xenon lamp and a 1-cm quartz cell. All of the spectral data were given with correction. The excitation and emission bands widths were set to 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-DBC was obtained from Fanghua Xueyuan Shiyan Huagongchang (China). The Britton-Robinson buffer was used to control the acidity of tested solutions.

Bovine serum albumin (BSA), human serum albumin (HSA), Human γ-IgG (IgG), egg albumin (Egg) and lysozyme (Lys) (Sigma, St Louis, MO, USA) were directly dissolved in water to prepare stock solutions and stored at 0–4 °C. The working solutions were obtained by diluting the stock solutions with water just prior to use.

All other chemicals were of analytical grade or the best grade commercially available. Human serum was provided by the First Hospital of Gansu Medical University and diluted 2000-fold with doubly deionized water.

General Procedures

1.0 ml of Britton-Robinson buffer, and an appropriate amount of the working solution of Arsenazo-DBC and protein (or sample) were mixed, then diluted to 10.0 ml with water and thoroughly stirred. 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 410.0 nm.

RESULTS AND DISCUSSION

Reaction and Spectra

The spectra of Arsenazo-DBC and Arsenazo-DBC-BSA at pH 4.10 are shown in Figure 2. As shown in Figure 2, the Arsenazo-DBC absorption spectrum changes slightly when BSA is added, while the RLS spectra changes significantly, which indicates some interaction between Arsenazo-DBC and proteins. Here it can be seen that the peak of light scattering of the complex does not appear within the widow of its absorption spectrum, so the reaction system could be treated as a transparent solution. This may be because the molecules simultaneously absorbs the incident light and the scattered light, resulting in a net overall weak light scattering intensity in the absorption region. Therefore, the quantitative basis for this assay is in accordance with the Rayleigh formula:[15]

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

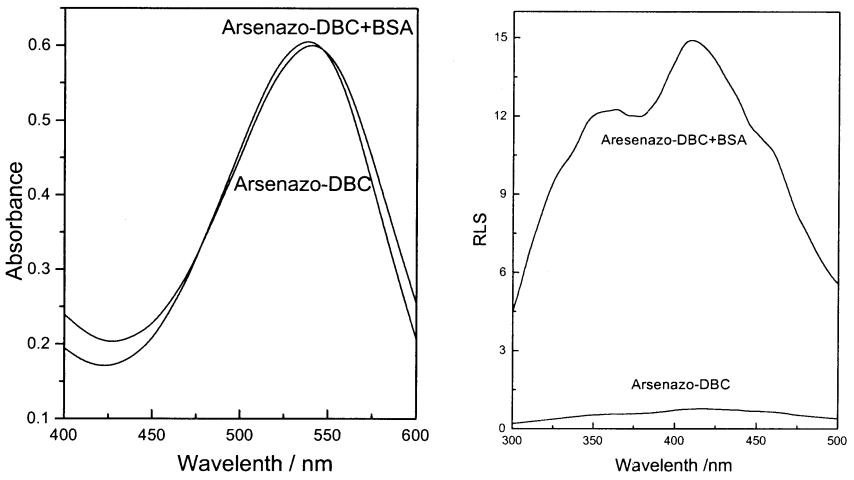

Figure 2. The absorption spectrum (Left) and resonance light scattering spectra (Right) for Arsenazo-DBC and the complex of Arsenazo-DBC-BSA.

where R_θ is the Rayleigh ratio at a 90° scattering angle (the Rayleigh ratio describes the scattering ability of the system), n_1 and n_2 are the refractive indices 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 volume/area. When the scattering species of the system is fixed, then v is constant. Because the scattering particle is an electrostatic Arsenazo-DBC-protein complex, the number of scattering particles is determined by the concentration of protein added at a fixed concentration of Arsenazo-DBC. Therefore N_0 is proportional to the concentration of the protein added, i.e. under otherwise fixed experimental conditions, the scattering light intensity I is determined only by the protein concentration C ($I = kC$).

Optimization of the General Procedure

It was found that the sequence of adding the reagents has some effects on the results of the sensitivity. In our paper, the procedure of reagent→buffer→protein was recommended in order to achieve high sensitivity and reproducibility.

To examine the effect of pH, its value was adjusted using the Britton-Robinson buffer and the scattering light was measured in the presence or

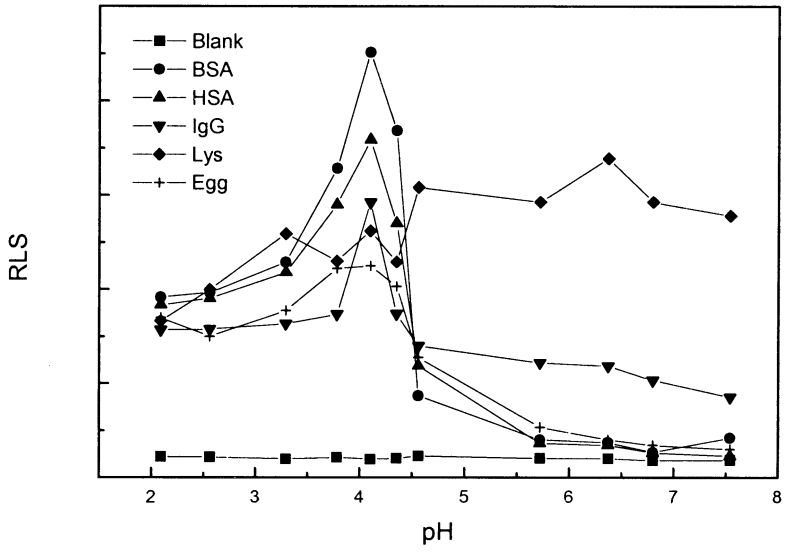

Figure 3. The dependence of RLS on pH. Concentration: Arsenazo-DBC, 25 μmol.l^{-1}; protein, 20.0 μg.ml^{-1}, λ = 410.0 nm.

absence of proteins. As shown in Figure 3, pH affects the RLS intensity significantly, due to the dissociation and ion charge of Arsenazo-DBC and proteins. The optimum pH conditions are found to be 3.78–4.35 for HSA, BSA, Egg, and IgG. For basic protein of Lys, in pH 3.78–7.54, the sensitivity is relatively well. In order to simplify the method and compare results of all proteins, pH 4.10 Britton-Robinson buffer is recommended for all experiments.

The effect of the concentration of the Arsenazo-DBC ($C_{Arsenazo\text{-}DBC}/\mu mol \cdot l^{-1}$) was tested, and the results were listed in Figure 4. It was found that the $C_{Arsenazo\text{-}DBC}$ affects the RLS sensitivity. When the $C_{Arsenazo\text{-}DBC}$ was low, the linear range was narrow, which may imply saturation binding of Arsenazo-DBC on the protein. When the $C_{Arsenazo\text{-}DBC}$ was high, the sensitivity decreases due to lower enhancement of the scattering. In order to get a higher sensitivity and a wider linear range, 25.0 $\mu mol \cdot l^{-1}$ Arsenazo-DBC was used.

The reaction was rapid, it only took about several seconds to reach the equilibrium of the reaction for either low or high levels of protein, and the intensity of scattering light remained relatively stable for at least 3 h.

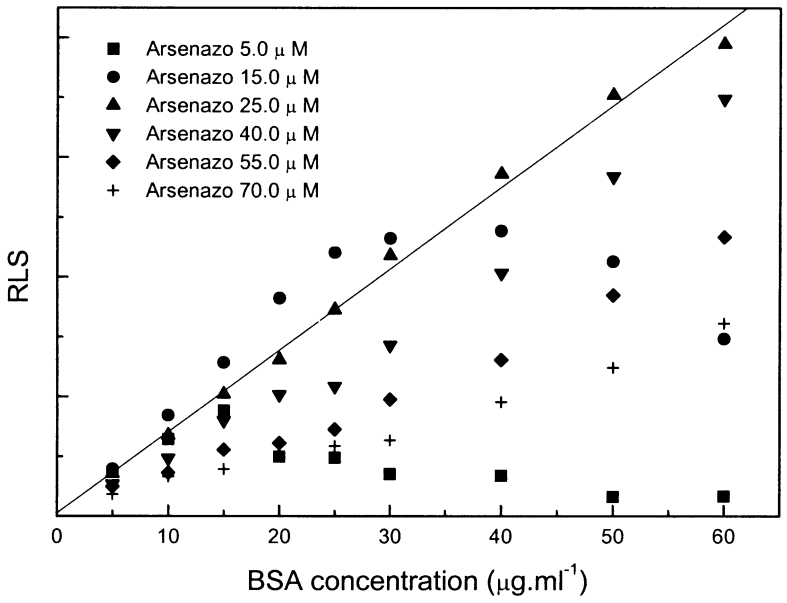

Figure 4. RLS response pattern of BSA under different concentrations of Arsenazo-DBC.

Calibration Graphs

Under the optimal conditions, the method has been used in the determination of several proteins, such as HSA, BSA, Egg, Lys and IgG. The standard regression equations for proteins are shown in Table 1. 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 determination is calculated by the following formulation:[16]

$$D_L = kS_{b1}/S \tag{2}$$

where D_L is the limit of detection; k is a constant related to the confidence level, according to the suggestion of IUPAC, $k = 3$; S_{b1} is the standard deviation of 5 blank measurements and S is the slope of the calibration graph.

Effect of the Ionic Strength

The interaction of proteins and Arsenazo-DBC is mainly as a result of electrostatic forces, so the ionic strength should affect the reaction. As shown in Figure 5, it has been found that the intensities of RLS are stable when the ionic strength is lower than 0.05 (NaCl). It decreases when the ionic strength is higher than 0.05. This phenomenon can possibly be ascribed to the decrease of the electrostatic force interaction between the Arsenazo-DBC and proteins, because of the shielding effect of the charges on proteins with increasing ionic strength, which is unfavorable to the formation of the complex.

Table 1. Standard Regression Equations for Proteins

Protein	Standard Regression Equation (C μg·ml^{-1})	Regression Coefficient	Linear Range (μg·ml^{-1})	Determination Limits (D_L) (ng·ml^{-1})
HSA	Y = −0.948+0.609 C	0.9971	2.5–60.0	98.50
BSA	Y = 0.267+0.681 C	0.9974	2.5–60.0	88.10
Egg	Y = 0.309+0.366 C	0.9978	2.5–60.0	163.9
Lys	Y = −0.944+0.410 C	0.9907	2.5–60.0	146.3
IgG	Y = −0.410+0.354 C	0.9991	2.5–60.0	169.5

Note: Arsenazo-DBC 25.0 μmol·l^{-1}, pH 4.10, average of five measurements.

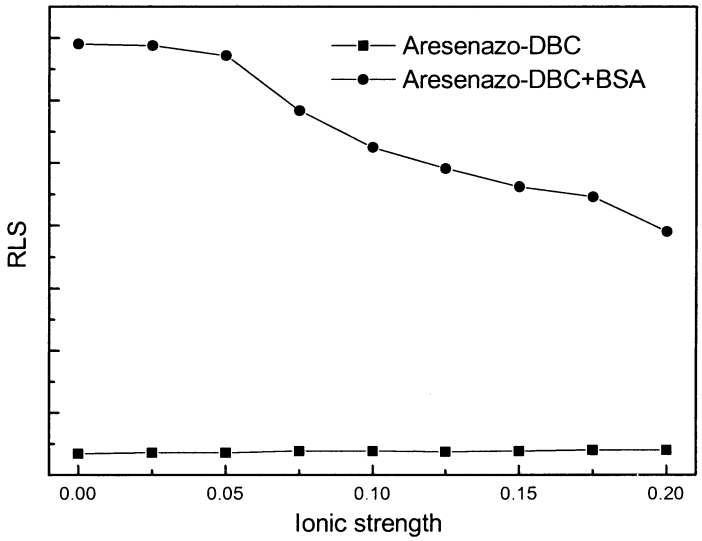

Figure 5. The effect of ionic strength on RLS of Aresenazo-DBC and Aresenazo-DBC-BSA. Arsenazo-DBC, 25 μmol·l^{-1}; protein, 20.0 μg·ml^{-1}, λ = 410.0 nm.

Interfering Substances

The influences of foreign coexisting substances were tested, and the results are shown in Table 2, As can be seen in this table, amino acids show little interference and few ions interfere with this assay, so no special preparation was needed prior to the serum sample determination.

Determination of Total Protein in Serum Samples

Since the present method showed protein specificity as revealed in the investigation, we thought it was suitable for determining the total content of protein in complex samples containing different types of proteins, e.g. serum samples. Thus this method was applied to the determination of total protein in human serum samples. The results were in good agreement with data provided by clinical physicians (listed in Table 3), indicating that this method has the potential for practical application to the determination of total protein.

Table 2. Effect of Interfering Substances

No.	Substance	Concentration ($\mu g \cdot ml^{-1}$)	Change of RLS Intensity (%)
1	Al^{3+}, chloride	20.0	29.21
2	Ca^{2+}, chloride	20.0	1.09
3	Cd^{2+}, chloride	20.0	0.90
4	Co^{2+}, chloride	20.0	0.53
5	Cr^{3+}, nitrate	20.0	8.45
6	Cu^{2+}, chloride	20.0	0.38
7	Hg^{2+}, chloride	20.0	−3.31
8	K^{+}, chloride	20.0	0.00
9	Mg^{2+}, chloride	20.0	1.86
10	Mn^{2+}, chloride	20.0	0.46
11	Na^{+}, chloride	20.0	0.01
12	Ni^{2+}, chloride	20.0	5.23
13	Ethanol	2.0%	0.23
14	Methanol	2.0%	0.42
15	Urea	40.0	0.16
16	Glucose	40.0	1.12
17	$H_2PO_4^{-}$,Na^{+}	20.0	0.13
18	CO_3^{2-}, Na^{+}	20.0	0.54
19	SO_4^{2-}, Na^{+}	20.0	−0.61
20	NO_3^{-}, Na^{+}	20.0	0.00
21	NO_2^{-}, Na^{+}	20.0	0.33
22	L-Asp	40.0	−0.01
23	L-Leu	40.0	0.00
24	L-Ala	40.0	1.00
25	L-Phe	40.0	0.00
26	L-Pro	40.0	0.48
27	L-Try	40.0	1.04
28	L-His	40.0	−0.54
29	L-Glu	40.0	1.04
30	L-Cys	40.0	−0.82
31	L-Arg	40.0	−0.14
32	L-Lys	40.0	0.57
33	L-Ser	40.0	−0.25
34	L-Thr	40.0	−0.83
35	L-Iso	40.0	0.00

Concentrations: BSA 20.0 $\mu g \cdot ml^{-1}$, Arsenazo-DBC 25.0 $\mu mol \cdot l^{-1}$, pH 4.10, average of five measurements.

Table 3. Analytical Results of Human Serum Samples

	Total Protein ($mg \cdot ml^{-1}$)			
	Sample I	Sample II	Sample III	Sample IV
Present method (n = 5)	68.35 (± 0.8)	69.55 (± 1.2)	72.11 (± 1.1)	69.06 (± 0.7)
Clinical data	68.40	72.50	70.70	70.90

CONCLUSION

The mechanism of the enhancement of RLS by proteins is still under study, but clearly this novel spectrophotometry can be used in protein determination. Compared with other general methods, the main advantage of the enhanced RLS technique is its high sensitivity and simplicity in handling in an ordinary biochemical laboratory.

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