Microdetermination of Proteins by Enhanced Rayleigh Light Scattering Spectroscopy with Thorin

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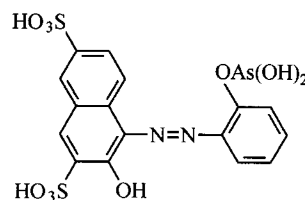
In acidic solution, thorin (2-(2-hydroxy-3.6-disulfo-1-naphthyl)-azo-phenylarsenic acid sodium salt) can be bound with protein and aggregated to form large particle which displays a very strong rayleigh light scattering (RLS). The effects of pH, thorin concentration, detergent and ionic strength on binding reaction have been studied. The interference of coexisting substances was checked. The Scatchard plots for reaction between thorin and bovine serum albumin (BSA) were constructed and the association constant of thorin-BSA was obtained, it is 5.26 × 10^5 L/mol, the maximum binding number is 7. RLS intensity is well proportional to the concentrations of 2.0—14.0 μg/mL for human serum albumin (HSA), 1.8—14.7 μg/mL for BSA and 1.8—14.6 μg/mL for γ-globulin (γ-G). The detection limits (3σ) are 54.1 ng/mL for HSA, 52.0 ng/mL for BSA and 51.8 ng/mL for γ-G, respectively. The relative standard deviation is 2.4% for BSA, 3.2% for HSA and 4.1% for γ-G. The human serum samples were measured satisfactorily by using this method.

Keywords thorin, protein, rayleigh light scattering

Introduction

Protein determination is always important in clinical applications. New methods including spcetrophotometry,[1] fluorometry[2] and chemiluminicence[3,4] are continuously being developed. Pasternack *et al*.[5,6] developed a technique to measure the intensity of scattering light using a common spectrofluorometer, through which the association of porphyrin with DNA and the aggregation of chlorophyll has been studied. With this technique, Huang *et al*.[7,8] established a sensitive method for determination of trace amount of DNA. Later, Ma *et al*.,[9,10] Yao *et al*.[11] and Wang *et al*.[12] applied this technique to protein assay. In this paper, a new method for protein assay by Rayleigh light scattering (RLS) with thorin was developed. It is simple, sensitive and rapid for protein determination.

Thorin is most frequently used as spectral analytical reagent and its structure is shown as follows:



It was found that the RLS intensity of thorin is greatly enhanced by proteins. Based on this observation, the protein in human plasma can be quantitatively detected. Interferences from coexisting substances, such as amino acids and metal ions, were investigated.

Experimental

Apparatus

RLS intensity and spectra were obtained from aqueous solution in 1-cm quartz cuvette using a Shimadzu Model RF-540 spectrofluorometer (Kyoto, Japan). A Shimadzu Model UV-265 double-beam spectrophotometer (Kyoto, Japan) was used for recording absorption spectra. pH values were measured with a Model 821 pH meter

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Reagents

Thorin was purchased from Beijing Chemical Plant (China). Protamine sulfate (Pro), bovine serum albumin (BSA), human serum albumin (HSA), bovine hemoglobin (Hb), γ-globulin (γ-G) and lysozyme (Lyso) were purchased from Sigma. Pepsin was provided by Shanghai Institute of Biochemistry.

The protein concentrations were determined by spectrophotometry at 280 nm using $\varepsilon^{1\%}$ values as follows: BSA, 6.6;[13,14] Lysozyme, 26.04;[15] HSA, 5.3;[16] γ-G, 13.8.[14] The concentrations of hemoglobin, protamine sulfate, pepsin were determined as follows:[17] protein concentration (μg/mL) = $144 \times (A_{215} - A_{225})$. A_{215} and A_{225} are the absorbance measured using a 1-cm cell at 215 nm and 225 nm, respectively.

All chemicals were of analytical reagents or the best grade commercially available. All solutions of proteins and chemicals were prepared in doubly deionized water. A series of Britton-Robinson buffer solutions (H_3PO_4 + HAc + H_3BO_3) was used for the pH adjustment.

Procedure

In a 10-mL volumetric flask, 2.0 mL of buffer solution (pH 3.73), 0.2—1.6 mL of BSA (100 μg/mL) standard solutions or 1.0 mL of specimen were mixed thoroughly. Then 0.5 mL of thorin (0.1%) solution was added and diluted to 10 mL with water and mixed thoroughly. Rayleigh light scattering spectra were scanned synchronously with the same wavelengths of excitation and emission ($\lambda_{ex} = \lambda_{em}$) by spectrofluorometer through the range of 300—700 nm at 5 nm slit-width. Based on these spectra, the RLS intensities were determined at 345 nm. The standard and unknown samples were prepared and run simultaneously under the same assay conditions.

Results and discussion

Absorption spectra and RLS spectra

The absorption spectra of thorin in the absence and presence of protein were shown in Fig. 1. The absorption difference between two BSA concentrations is too small to explore a relatively sensitive procedure. However, we can find that the case is different for RLS spectra and intensity.

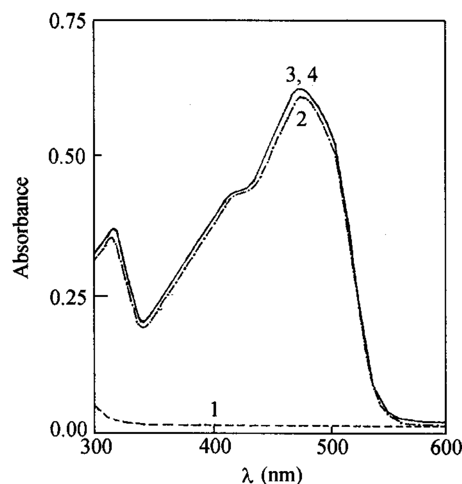

Fig. 1 Absorption spectra at pH 3.73 1) BSA (10 μg/mL); 2) thorin (0.005%); 3) thorin-BSA (5 μg/mL); 4) thorin-BSA (10 μg/mL).

Fig. 2 shows that the spectra of thorin has two peaks at 345 nm and 570 nm, respectively. The protein has a wide bands over range of 300—500 nm with relatively small intensity. When protein coexists in the system, the RLS intensity of thorin has a significant increase and the increase at extent 345 nm is greater than that at 570 nm, so 345 nm was selected as the determination wavelength.

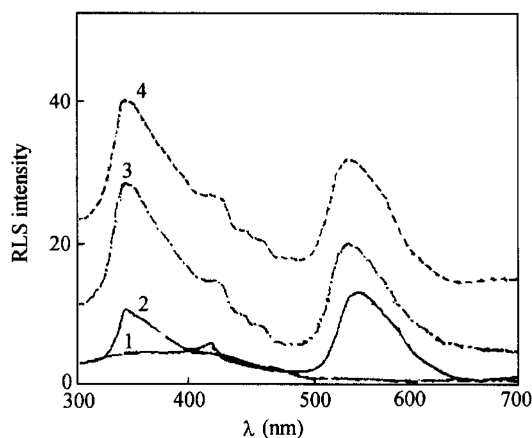

Fig. 2 RLS spectra at pH 3.73 1) BSA (10 μg/mL); 2) thorin (0.005%); 3) thorin-BSA (5 μg/mL); 4) thorin-BSA (10 μg/mL).

Optimization of experimental condition

The reaction between thorin and BSA occurred rapidly at room temperature (< 5 min). The scattering

intensity is stable for at least 4 h. Three kinds of mixing orders were investigated. The results revealed that BSA should be mixed with buffer first so that BSA can be protonized. The positively charged BSA interacts with thorin through electrostatic forces.

Effect of pH

The effect of pH on RLS was investigated from pH values of 1.75—5.72. The results revealed that the scattering intensity of the assay system was greatly affected by pH. The relative scattering intensity reaches its stable maximum at pH values of 3.50—3.95, after that RLS intensity sharply decreased. pH value of 3.73 was chosen for the assay.

Effect of thorin concentration

The effect of thorin concentration (0.003%—0.008%) on scattering intensity was studied with the BSA (2.5—15.0 μg/mL) standard assay. The desired linear range and RLS intensity could be obtained with thorin concentration 0.005%.

Effect of detergent

Cetyltrimethylammonium bromide (CTAB) increased the RLS intensities of both the reagent blank and complex, and the signal of the reagent blank is greater than that of the thorin-BSA system when CTAB concentration is above 0.0035%. This may be explained by the fact that positively charged CTAB competed with BSA and formed a thorin-CTAB associate,[18] which was insoluble in aqueous solution and gave enormous scattering signal. Detergents such as SDBS, Triton X-100, β-CD slightly increased the RLS intensity in the assay, but they did not change the reagent blank.

Effect of ionic strength

The effect of NaCl content on this ssay was examined at pH 3.73 and proved to have significant effect on the interaction. The relative RLS intensity decreased with increasing salt concentration (Fig. 3). When the content of NaCl increases, the electrostatic shielding of charges reduced the binding between the dye and the BSA, and result in a decreased signal.

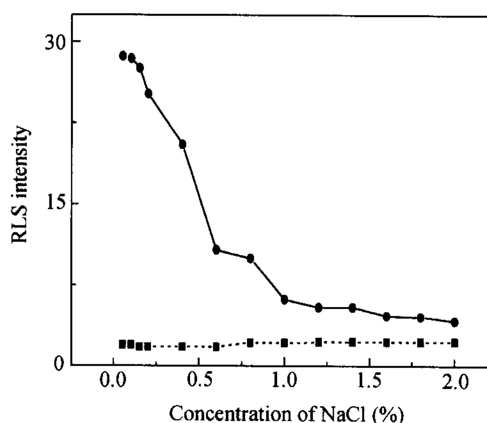

Fig. 3 Effect of ionic strength on scattering intensity of thorin (■) and thorin-BSA (●) (thorin 0.005%).

Effects of interfering substances

The interference of coexisting substances, such as amino acids and metal ions, glucose, nucleic acid, urea *et al*. was tested. The results were presented in Table 1. Most substances do not interfere appreciably with this assay.

Table 1 Foreign substances effects on the RLS method for proteins determination

Substance	Concentration (μg/mL)[a]	Change in RLS intensity (%)[b]
Gly	3.34	-4.9
Leu	12.36	10.02
Ser	23.6	0.94
Cys	12.3	4.2
Asp	12.5	5.8
Glu	21.1	4.4
Lys	17.7	3.8
Phe	2.9	7.4
Try	6.2	-4.6
Tyr	5.65	-5.3
Ala	12.2	0.98
Arg	19.6	8.2
Al(III), SO_4^{2-}	2.7	0.00
Fe(III), Cl^-	8.94	-3.9
Mg(II), SO_4^{2-}	2.43	2.4
Mn(II), Cl^-	5.49	4.1
Co(II), Cl^-	5.89	-7.0
Ca(II), Cl^- P	12.02	4.3

Continued

Substance	Concentration (μg/mL)[a]	Change in RLS intensity (%)[b]
Cd(II), Cl^-	11.24	1.2
Cr(III), NO_3^-	5.2	7.2
Cu(II), SO_4^{2-}	6.35	5.3
Zn(II), Cl^-	6.5	1.9
Ni(II), Cl^-	0.59	8.7
Pb(II), NO_3^-	2.07	-6.8
EDTA	374	-2.2
Glucose	1.8	1.83
Lactose	46.12	-3.94
Sucrose	10.0	-3.10
CtDNA	12.94	-0.84
Uracil	11.2	3.54
Cytidine	73.94	-4.89
Adenosine	26.72	7.58
Urea	12.01	-0.84

[a] Average value from at least two measurements. [b] BSA: 5.0 μg/mL; thorin: 0.005%. All values were obtained by the standard procedure at pH 3.73.

Standard regression equation for proteins

The standard regression equations for variant proteins were shown in Table 2. Different proteins have different isoelectric points. At the same time, the weight, size and shape of the molecules are also various, so the RLS signals for various proteins varied. Pepsin showed no scattering enhancement toward thorin. The linear ranges are 2.0—14.0 μ/mL, 1.8—14.7 μg/mL and 1.8—14.6 μg/mL for HSA, BSA and γ—G, respectively. The sensitivity of thorin method for protein determination is obviously higher than that of common protein assay.

Calculation of association constant of thorin-BSA

Yang[19] obtained formula from the Scatchard equation:[20]

$$F/[L] = F_\infty/K_d - F/K_d$$

Where [L] is the free dye concentration and K_d is the dissociation constant. F is the measured fluorescence intensity and F_∞ is the fluorescence intensity in the presence of infinite dye concentration (extension value). Considering that fluorescence and RLS are based on the same theory, and $F = kc$ as well as $I = kc$ (where F is fluorescence intensity, I is RLS intensity and c is the concentration of protein), that is, RLS intensity and fluorescence intensity are proportional to the content of proteins, we substitute I_{RLS} and I_∞ for the F and F_∞ of Scatchard equation:

$$I_{RLS}/[L] = I_\infty/K_d - I_{RLS}/K_d$$

Where I_{RLS} is the measured RLS intensity and I_∞ is the RLS intensity in presence of infinite thorin concentration. When BSA concentration was low enough, the bound thorin was insignificant. [L] was approximately equal to the total thorin concentration. Knowledge of I and [L] in solution allows calculation of K_d which may be found from the slope of a linear plot of $I_{RLS}/[L]$ versus I, I_∞ can also be obtained from the intercept of the plot (Fig. 4).

Table 2 Analytical parameters of RLS method for proteins determination

Protein	Standard regression Equation (ρ/(μg/mL))	Linear range (μg/mL)	r^a	DL (3σ, ng/mL)	RSD (%)[b] (n = 7)
HSA	$\Delta I = 2.34 + 3.12\rho$	2.0 - 14.0	0.997	54.1	3.21
BSA	$\Delta I = 2.37 + 4.80\rho$	1.84—14.7	0.998	52.0	2.40
γ-G	$\Delta I = 3.77 + 1.17\rho$	1.83—14.6	0.985	51.8	4.07
Lyso	$\Delta I = 4.08 + 2.30\rho$	1.7—13.6	0.976	47.0	2.93
Hb	$\Delta I = 5.47 + 2.78\rho$	1.4—11.4	0.986	39.2	4.78
Pro	$\Delta I = 5.91 + 7.07\rho$	0.87—6.96	0.987	20.7	5.16

[a] Regression coefficient. [b] Relative standard deviation for seven measurements of protein (5.0 μg/mL).

A series of assay mixtures was made up to contain a constant total BSA concentration of 5.0 μg/mL in a 10 mL-final volume. These mixtures varied in thorin content from 1.67×10^{-5} mol/L to 10.02×10^{-5} mol/L. From the linear plot of IRLS/[L] versus I, the association constant ($1/K_d$) was calculated. The value is 5.26×10^5 L/mol. The maximum binding number is 7.

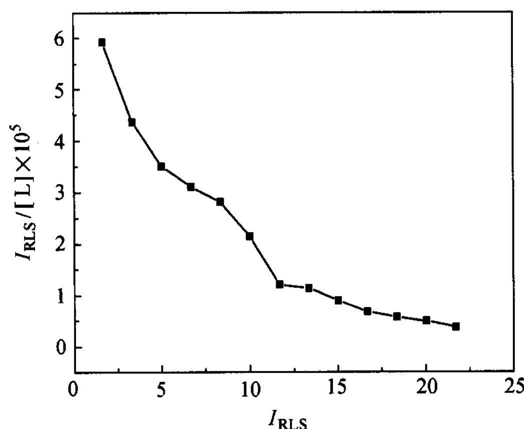

Fig. 4 Scatchard plot of thorin binding to BSA.

Sample determination

BSA is similar to HSA in terms of structure and biological function, and less expensive. Therefore BSA was used to optimize the condition for the determination, and HSA was used for the standard curve. Seven samples were assayed according to the general procedure, and the results were compared with Commassie Brilliant Blue (CBB) method,[21] which is widely used in clinical application (Table 3). The results obtained with the thorin method are close to those obtained with the CBB method. The stability of thorin method is higher than CBB method as well as easier washing of the tubes and cuvette because Commassie brilliant adheres to the glass.

Table 3 Comparison of the thorin assay and the CBB assay for protein in human plasma[a] (from adults)

Serum sample	Protein (mg/mL, found in human plasma)	
	Thorin assay	CBB assay
1	75.2	74.1
2	75.2	70.0
3	79.6	72.6
4	79.0	78.4
5	73.2	75.4
6	75.4	75.6
7	72.6	76.8

[a] obtained from the Hospital of Peking University.

Conclusion

Thorin is an acid azo dye. A thorin molecule has hydrophilic sulfonic groups. At pH 3.73, thorin is negatively charged (H_4L^-, H_4L^{2-}) and protein is positively charged, since isoelectric points of BSA and HSA are 4.6—4.7 and 4.8, respectively. The dissociated sulfonic group in thorin can interact with positively charged groups in protein, such as protonated amino groups. This makes thorin and protein bind through electrostatic force. Meanwhile, the aromatic rings of thorin and protein have interaction due to non-electrostatic effects, such as hydrophobic and Van der Waals force, which leads to the aggregation of thorin molecules on the surfaces of protein molecule. The aggregation will produce relatively large particles, and results in the enhancement effect of protein on RLS intensity of thorin.

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