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A rapid and sensitive method for the determination of trace proteins based on the interaction between proteins and Ponceau 4R

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Abstract

The interactions between proteins and Ponceau 4R (PR) in aqueous solution have been studied by the techniques of resonance light scattering (RLS) spectroscopy, the absorption spectroscopy, zeta potential assay and circular dichroism (CD) spectrum. The dry PR can assemble on the surface of protein via electrostatic and hydrophobic forces to produce an associated compound of protein-PR, this compound can enhance the RLS of protein. Based on this fact, a simple, rapid, and sensitive method has been developed for the determination of proteins at nanogram level by RLS technique with a common spectrofluorimeter. Under optimum conditions, the linear range is 0.10–39.2 $\mu g\,mL^{-1}$ for the determination of both bovine serum albumin (BSA) and human serum albumin (HSA). The detection limits (S/N = 3) are 6.96 $ng\,mL^{-1}$ for BSA and 5.71 $ng\,mL^{-1}$ for HSA, respectively. There is almost no interference from amino acids, most of the metal ions, and other coexistent substances. The method has been satisfactorily applied to the direct determination of the total protein in human serum.

Keywords: Resonance light scattering; Protein; Ponceau 4R; Determination

1. Introduction

The study on the interaction between small molecules and proteins is important in biochemistry. Since organic dyes can be used as effective probes of the structure and function of biological macromolecules and as the study models of some biophysical processes [1,2], there has been an increasing interest in the studies of the interactions between organic dyes and biological macromolecules [3–5]. Based on these interactions, a lot of analytical methods for biological macromolecules can be established. The most widely accepted assays so far for proteins are the Lowry [6], Bradford [7,8], Bromophenol Blue [9] and Bromocresol Green [10] methods. However, most of these methods suffer from the disadvantages of low sensitivity. Pasternack et al. [11,12] used a common spectrofluorimeter to study the aggregation of porphyrins and found that the intensity of the resonance light scattering was proportional to the concentration of DNA within a certain concentration range. Later on, Huang et al. discovered that by using the enhanced RLS intensity, trace amounts of nucleic acids [13,14] and proteins [15] in synthetic and practical samples could be sensitively determined. The light scattering technique has become a new interesting method for the determination of biomacromolecules [16–24] for it could be performed by an ordinary fluorescence spectrometer and the detection limit of biomacromolecules could be quite low.

The (4-sulpho-1′-naphthylazo)-1-hydroxy-2-naphthalene-6,8-disulphonic acid trisodium salt (CI 16255) known as Ponceau 4R (PR, see Fig. 1) [25] is a synthetic organic azo group food colorant that can be found in common food products such as beverages, dry mix products, candies, dairy products, sugar confectioneries and bakery products. However, up to now, using it for the determination of proteins has not yet been paid much attention to. In this paper, we firstly studied the characteristics of RLS spectra of PR. In weakly acidic solution, proteins can enhance

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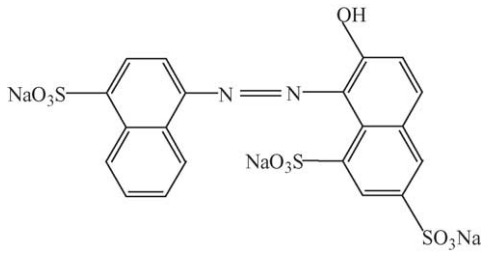

Fig. 1. The structure of Ponceau 4R.

the RLS intensity of PR. The enhanced RLS intensity is proportional to the concentration of proteins, thus some proteins can be determined. Especially, the detection limits for bovine serum albumin (BSA), human serum albumin (HSA) and human immunoglobulin G (IgG) are all down to10.0 ng mL^{-1}. Little interference from most co-existing substances was observed. The presented method was applied to the determination of proteins in human serum samples, and the results were close to those provided by clinical physicians (Biuret Colorimetric method). The mechanism of interaction between PR and proteins is also studied by absorption spectroscopy, zeta potential assay and circular dichroism (CD) spectrum.

2. Experimental

2.1. Apparatus

The intensity and spectra of RLS were obtained with a Hitachi 850 (Japan) and a Perkin-Elmer LS55 fluorescence spectrophotometer (UK), respectively. A quartz cassette (1 cm × 1 cm cross-section) was used in the experiments. The absorption spectra were obtained by Shimatzu UV-240 spectrophotometer (Japan). Zeta potential assay was obtained by 90 Plus Particle Size Analyzer (Brooknaven, USA) and circular dichroism (CD) spectrum was obtained by Model J-810 spectropolarimeter (Jasco, Japan). All pH measurements were made with a pH-II acidity meter (Leici factory, Shanghai, China).

2.2. Reagents

All chemicals were of analytical reagent grade. Bovine serum albumin (BSA) (Hua-Mei Biochemical Reagent Co., China) and human serum albumin (HSA), egg albumin, human IgG, myoglobin, hemoglobin and lysozyme (Sigma St. Louis, MO, USA) were directly dissolved in water to prepare stock solutions and stored at 0–4 °C. The accurate concentrations were determined spectrophotometrically at 280 nm with the $\varepsilon^{1\%}$ values.

A 2.2×10^{-2}% working solution of PR (Shanghai Dyestuffs Research Institute Co., Ltd, China) was prepared for use.

Three buffer solutions of 0.1 mol L^{-1} Sϕrensen ($C_6H_5O_7Na_3$–NaOH), 0.2 mol L^{-1} Clark–Lubs ($KHC_8H_4O_4$–HCl) and 0.4 mol L^{-1} Britton–Robinson (H_3PO_4–HAc–H_3BO_3–NaOH) were used in the experiments.

Fresh Human serum was obtained from the Hospital of Nanjing University. All stock solutions were prepared in doubly de-ionized water.

2.3. Procedures and methods

An appropriate volume of working solution of proteins, 2.0 mL of B–R buffer solution and an appropriate PR solution were added into a 10 mLvolumetric flask in turn and mixed thoroughly. The mixture was diluted to 10 mL with doubly de-ionized water. The RLS spectra were recorded by scanning simultaneously with same wavelengths of excitation and emission ($\lambda_{ex} = \lambda_{em}$) from 300 to 700 nm on the spectrofluorimeter (with 5 nm slit-width) and the RLS intensity I for the reaction product and I_0 for the reagent blank were measured at the maximum scattered wavelength, $\Delta I = I - I_0$.

The circular dichroism (CD) spectra of the associated compound were recorded at 25 °C on a Model J-810 spectropolarimeter (Jasco, Japan). Solutions of PR, BSA and their complexes in B–R buffer (pH 2.5) were scanned in 1.00 cm quartz cell at 0.2 nm intervals, with three scans averaged for each CD spectrum in the range of 200–300 nm. Results were expressed in molar ellipticities [θ]. The results are expressed as molar ellipticities $[\theta]_\lambda$ in ° cm^2 $dmol^{-1}$ which is defined as [26,27]:

$$[\theta]_\lambda = \frac{10h}{cd}$$

where h is the observed ellipticity in degrees, c, the concentration in mol L^{-1} of total bilirubin and d, the pathlength in dm.

3. Results and discussion

3.1. The interaction between protein and PR

In order to understand the binding force and to study the interaction between BSA and PR, Rayleigh light scattering spectroscopy, the absorption spectroscopy, zeta potential assay and circular dichroism spectrum methods are used in this work. The light scattering spectra and absorption spectra for PR and BSA-PR at pH 2.5 are shown in Fig. 2(A) and (B). The absorption spectra changed slightly when BSA was added to PR, while the RLS spectra greatly changed, which indicated that an interaction between proteins and PR occurred. As well known, protein folding is influenced by many forces and associated with related interactions, such as hydrophobic interactions, electrostatic interactions (charge repulsion and ion pairing), hydrogen bonding interactions, intrinsic propensities, and van der Waals forces, etc. Hydrophobic interactions are the repulsion between water and non-polar residues in

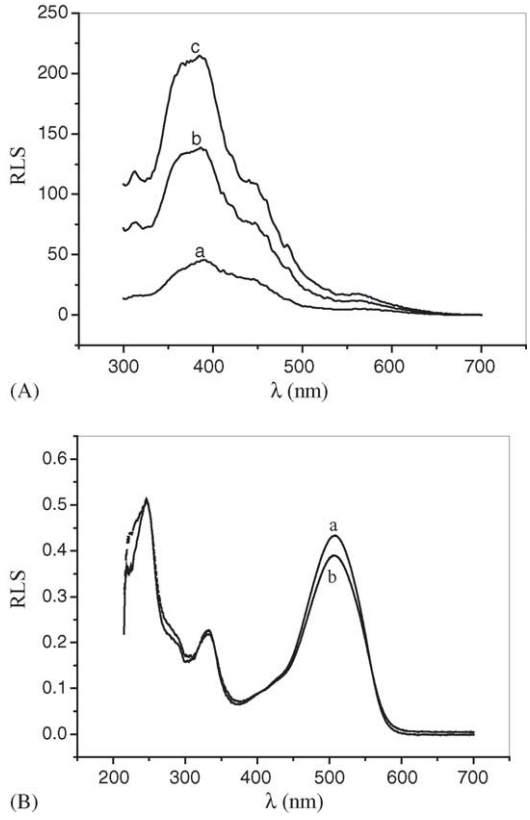

Fig. 2. (A) RLS spectra of PR-BSA. BSA: (a) 0.0 μg mL^{-1}; (b) 10.0 μg mL^{-1}; (c) 20.0 μg mL^{-1}; PR: 2.2×10^{-3}%; pH 2.5. (B) Absorption spectra of PR-BSA. BSA: (a) 0.0 μg mL^{-1}; (b) 10.0 μg mL^{-1}; PR: 2.2×10^{-3}%; pH 2.5.

proteins, leading to minimal hydration of the hydrophobic core. These interactions are strongly disfavored and associated with a large increase in heat capacity [28,29].

Fig. 3 showed the values of ζ of BSA-PR system at various concentrations of BSA. It can be seen that the ζ of PR (2.2×10^{-3}%) system is about -22.48 mV. With the addition of BSA to this system, the values of ζ increase and reach 0 mV at 4.0 μg mL^{-1} (=5.5×10^{-8} mol L^{-1}) BSA. Further addition of BSA will result in positive values of ζ. Noticing that the isoelectric point of BSA is 4.7 [30], when pH is

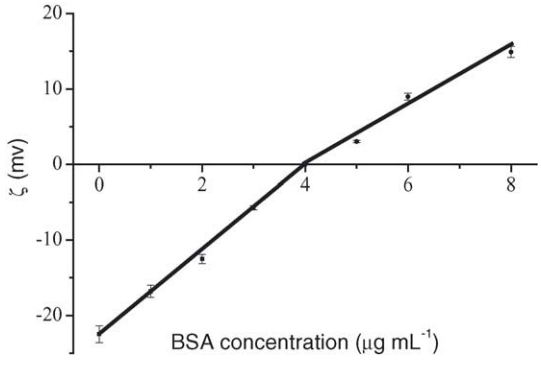

Fig. 3. Effect of concentration of BSA on ζ (mV) PR 2.2×10^{-3}%; pH 2.5.

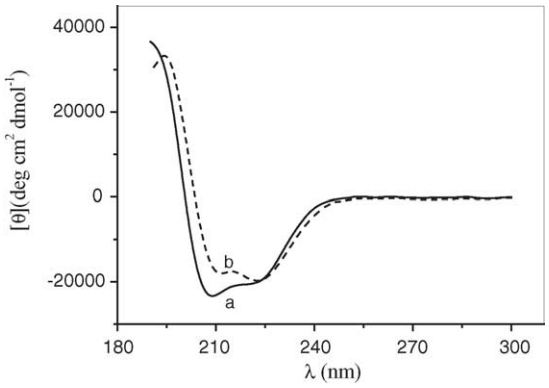

Fig. 4. CD spectra of PR-BSA system: (a) BSA; (b) BSA + PB. PR: 2.2×10^{-3}%; pH 2.5; BSA: 5.0 μg mL^{-1}.

changed to lower than 4.7, BSA is positively charged, and it would bring about the interaction between the positively charged BSA and the negatively charged PR. No doubt the PR molecules would bind BSA molecules through electrostatic attraction to form the associated compound of PR-proteins.

In order to further demonstrate the interaction mechanism, CD spectral data are used to offer the evidence about the binding of PR to BSA. As shown in Fig. 4, the double minimum peaks in the range between 200 and 230 nm indicate that BSA contains mainly α-helices structure in native BSA. The intensities of the double minimum peaks reflect the amount of helices of BSA, and the change in the intensity of these spectra bands reveals the change of the α-helices content in proteins [31,32]. In comparison with curve a and curve b, the obvious difference in shape and intensity of minimum peak near ca.209 nm can be observed. The wavelength of minimum at curve b in the presence of PR is shifted from 209 to 211 nm, and its intensity also greatly decreased in comparison with curve a. According to the decrease of intensity, the reduction of protein helices is estimated from Fig. 4 to be ca. 20% (66.8% content in native BSA, and 46.8% content after the binding of PR to BSA), which also implies that the binding of PR to BSA might induce some conformational changes. The results suggest that hydrophobic interactions also occur between PR and BSA besides electrostatic forces.

The formation of associated compound of protein-PR results in RLS enhancement. The RLS intensity is stable at least for 6 h, and the reaction between PR and proteins occurs immediately at room temperature (<2 min). Grounded on that the weak RLS of PR could be greatly enhanced in the presence of trace proteins, a new method for the determination of trace proteins is established. In optimal experimental conditions, the maximum RLS peak is located at 376.0 nm and selected in the assay.

3.2. The optimum conditions of protein determination

3.2.1. Effect of addition sequence and pH

Different addition orders of reagents have obviously different effect on the interaction of PR and BSA. Two types

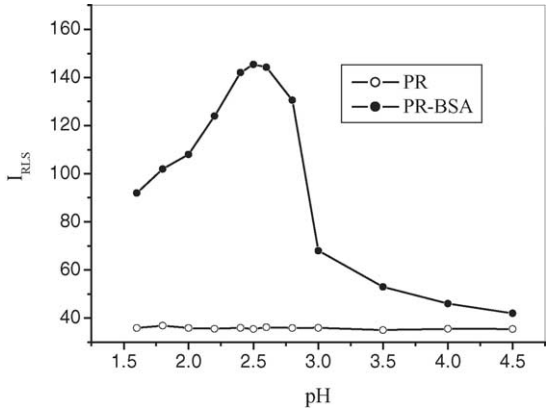

Fig. 5. Effect of pH on RLS intensity of PR-BSA. BSA: 5.0 μg mL^{-1}; PR: 2.2×10^{-3}%; pH: 2.5.

of mixing sequences were investigated. It is found that if the order of addition reagent is buffer, protein and PR, both the stability and intensities of RLS signals are the better. Mixing the buffer and protein at first can provide proper acid condition for their combination and improve the sensitivity. When the proteins and PR are mixed firstly, the solution pH is nearly neutral, which does not facilitate their combination.

We investigated three buffer solutions of HCl–NaAc, Clark–Lubs (HCl–$KHC_8H_4O_4$) and Britton–Robinson [(H_3PO_4, HAc, H_3BO_3)–NaOH] and found that the sensitivity and stability of the Britton–Robinson buffer solution was the best. So, the Britton–Robinson was selected. The scattering intensity of the assay system was greatly affected by pH, while the RLS of PR remained unchanged (Fig. 5). At pH 2.5, the scattering intensity reaches its maximum, so this pH was run for the assay.

3.2.2. Effect of PR concentration

The effect of PR concentration was shown in Fig. 6. It can be seen that the concentration of PR affects intensities of the RLS, and more intensity and widest linear range were reached at a PR concentration of 2.2×10^{-3}%. Hence, this concentration was chosen for the assay.

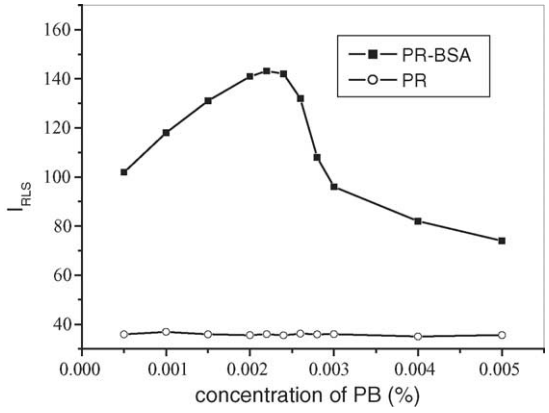

Fig. 6. Effect of PR concentration on this assay at pH 2.50. BSA: 5.0 μg mL^{-1}.

Table 1
Effect of potentially interfering substances on the reaction of PR with BSA

Interfering substances	Change (%)	Interfering substances	Change (%)
L-Arginine	−2.5	Al^{3+}(sulfate)	6.7
L-Cysteine	−2.2	Mn^{2+}(sulfate)	3.2
L-Glycine	−1.7	Pb^{2+}(nitrate)	3.6
L-Lysine	−3.3	Cr^{3+}(chloride)	−0.22
L-Glutamic	0.62	Cu^{2+}(nitrate)	1.9
L-Tryptophan	−6.3	Co^{2+}(chloride)	2.3
L-Serine	4.5.	Mg^{2+}(nitrate)	3.9
L-Leucine	2.1	Zn^{2+}(chloride)	4.8
α-Alanine	2.3	Ca^{2+}(nitrate)	0.12
β-Alanine	5.2	Fe^{3+}(nitrate)[a]	6.2
L-Tyrosine	6.7	Ni^{2+}(chloride)	4.2
L-Asparagine	7.8	Cd^{2+}(nitrate)	−2.7
ctDNA[b]	5.7		

Concentration of PR: 2.2×10^{-3}%; pH 2.5; concentration of interfering substances was 20.0 μmol L^{-1}; BSA: 5.0 μg mL^{-1}.
[a] Concentration of Fe^{3+} was 20 μg mL^{-1}.
[b] ctDNA concentration was 0.4 μg mL^{-1}.

3.2.3. Effect of interfering substances

Under the optimum conditions, the influence of coexisting substances, such as amino acids, metal ions and detergents, is tested and the results are shown in Table 1. It can be seen that amino acids and most of these metal ions hardly interfere with this assay and can be allowed with very high concentrations under the tolerance level of 10%. Although Fe(III) and DNA can be allowed at somewhat low concentrations (20 and 0.4 μg mL^{-1}, respectively), their concentrations are still higher than that in human plasma. Therefore, no special treatment was required before the sample analysis.

Fig. 7 shows the effects of surfactants such as sodium dodecyl sulphate (SDS), Tween-20, cethyltrimethylammonium bromide (CTAB) and β-cyclodextrin (β-CD). Tween-20, CTAB, and β-CD do not affect the RLS of PB and they almost have the similar effect for PR-BSA complex, but SDS is different. SDS leads to an increase in RLS intensity since negatively charged SDS binding with BSA-PR complex resulted in the increase of scattering intensity.

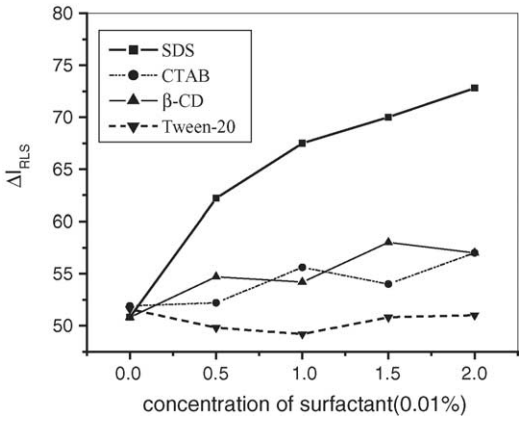

Fig. 7. Effect of surfactant on the RLS intensity of PR-BSA. pH 2.50. PR: 2.2×10^{-3}%; BSA: 5.0 μg mL^{-1}.

Table 2
Regression equation of proteins[a]

Proteins	Regression equation	Correlation coefficient	Linear range ($\mu g\,mL^{-1}$)	Detection limit[b] ($ng\,mL^{-1}$)	p*I*
BSA	$\Delta I = 9.65 + 9.47c$	0.9994	0.10–39.2	6.96	4.8–4.9
HAS	$\Delta I = 8.62 + 9.33c$	0.9996	0.10–39.2	5.71	4.7
IgG	$\Delta I = 9.90 + 9.31c$	0.9995	0.10–39.2	5.75	5.8–6.6
Lysozyme	$\Delta I = 14.1 + 3.67c$	0.9980	0.10–24.4	17.8	11–11.2
Hemoglobin	$\Delta I = 12.4 + 8.89c$	0.9994	0.10–29.4	13.3	6.9
Egg albumin	$\Delta I = 10.7 + 8.93c$	0.9994	0.20–34.4	9.9	4.6–4.7
Myoglobin	$\Delta I = 9.13 + 9.25c$	0.9995	0.10–39.2	7.2	6.8–7.0

[a] Concentration of PR: 2.2×10^{-3}%; pH 2.5.
[b] Calculated from three times the S.D. of 11 blank measurements.

Table 3
Comparison of the detection limit of protein with various probes

Probe[a]	Protein	Detection limit ($ng\,mL^{-1}$)	References
Ponceau G	BSA/HAS	14.9/5.0	[17]
Morin-CTMAB	BSA/HAS	66.0/23.0	[18]
Eriochrome Blue Black R	BSA/HAS	33.0/25.0	[19]
Orange G	BSA/HAS	2.6/3.4	[20]
Arsenazo DBN	BSA/HAS	67.4/44.8	[21]
This probe	BSA/HAS	6.96/5.71	

[a] Probes based on light scattering technique.

3.3. Calibration curves and sample determination

The individual calibration equations of various proteins assayed in the system are shown in Table 2. The limits of detection (LOD) were given by the equation $LOD = KS_0/S$, where K is a numerical factor chosen according to the confidence level desired, S_0 is the standard deviation (S.D.) of the blank measurements ($n = 10$, $K = 3$) and S is the slope of the calibration curve. The R.S.D. for 11 determinations of 1.0 $\mu g\,mL^{-1}$ HSA was 2.0%. Satisfactory linear relationships and low determination limits were obtained. Different proteins have different isoelectric points, and the size, mass and shape of the molecules are also different, and so the sensitivity of RLS for various proteins is different. Compared with other probes, Ponceau 4R shows good performance in determining proteins (Table 3).

Human serum samples, obtained from the Hospital of Nanjing University, were stored at 0–4 °C and diluted 1000-fold with de-ionized water just before the determination. By using the present method, total proteins in human serum samples were determined. The results are shown in Table 4. Compared with those by the clinical data, the present results are very satisfactory, indicating the potentiality of the presented method in clinical testing.

Table 4
The results for the determination of protein in human serum[a]

Sample	The presented method ($mg\,mL^{-1}$)	R.S.D.	Clinical data ($mg\,mL^{-1}$)
1	71.2	2.6	71.6
2	73.5	1.3	73.6
3	76.8	2.1	76.7
4	77.8	3.6	77.6
5	80.6	2.8	80.2

[a] Each result was the average of six measurements.

4. Conclusion

Based on the fact that the weak resonance light scattering intensity of Ponceau 4R is greatly enhanced in the presence of proteins, we developed a new method for trace protein determination at nanogram level. The presented PR method is sensitive, simple and convenient. It can be applied to the determination of total protein in human serum samples. All the results are satisfactory. The reaction mechanism is studied by zeta potential assay and circular dichroism (CD) spectrum. The obtained results suggest that the interaction between PR and protein via electrostatic and hydrophobic forces results in the formation of large associate of protein-PR and RLS enhancement of protein.

Acknowledgements

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