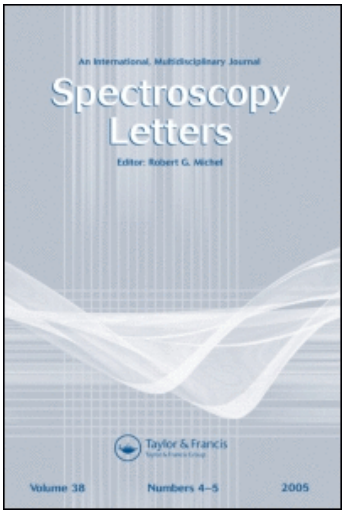

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Determination of Microamounts of Proteins by Resonance Light Scattering with Copper Phthalocyanine Tetrasulfonic Acid

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Abstract: Based on the strong enhancement effect of proteins on the resonance light scattering of copper phthalocyanine tetrasulfonic acid, a method for the determination of microamounts of proteins has been developed. Under the experimental conditions (2.0×10^{-6} mol/L copper phthalocyanine tetrasulfonic acid, pH 2.60, ionic strength 0.001 mol/L NaCl), the linear range of this assay is 0.06–4.0 μg/mL for bovine serum albumin (BSA), 0.1–2.0 μg/mL for human serum albumin (HSA), 0.0–2.0 μg/mL for human γ-IgG, and 0.2–6.0 μg/mL for ovalbumin. The detection limits (3δ) are 16.8 ng/mL for BSA, 23.4 ng/mL for HSA, 37.6 ng/mL for human γ-IgG, and 48.3 ng/mL for ovalbumin, respectively. This method has been applied to the analysis of total proteins in human serum samples collected from the hospital, and the results were in good agreement with those reported by the hospital.

Keywords: Copper phthalocyanine tetrasulfonic acid, proteins, resonance light scattering

INTRODUCTION

The development of novel methods and new techniques of protein determination is very important for both clinical and other laboratory tests. Traditional methods for protein assay are the Lowry method[1] and the Bradford method.[2] Also, some spectrophotometric methods,[3–5] fluorometric methods,[6–8] and

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chemiluminescence methods[9–11] have been developed. Resonance light scattering (RLS) has been widely studied and applied to solve many problems in recent years for its high sensitivity, simplicity, and quickness. The determination is mainly based on the fact that the aggregation of dye chromophore on the biological macromolecule can give rise to strong RLS. Tong and his co-workers[12] first developed the RLS technique for determining the concentration of nucleic acids. Also, methods for the determination of proteins in aqueous solution have been developed based on the enhancement effect of proteins on the RLS of organic dyes such as acid chrome blue K,[13] alcian blue 8GX,[14] acid green 25,[15] sulfonazo^{3+},[16] and 4-azochromotropic acid phenylfluorone.[17] Hence, resonance light scattering has become an interesting new method for the determination of microamounts of biomacromolecules.

Phthalocyanine is a well-known macrocyclic molecule featuring an 18-π conjugated electron system that inparts extremely high chemical and thermal stability to the macrocycle.[17–19] Nonsubstituted phthalocyanines are insoluble in common solvents such as water or ethanol, whereas those with substituent groups such as sulfo and carboxyl in the periphery of the macrocycle become soluble. Xu et al.[20] have established a sensitive RLS method for the determination of proteins using tetra-substituted sulfonated aluminum phthalocyanine, but they only supplied a method for the determination of two kinds of protein. In this work, a water-soluble derivative of copper phthalocyanine, copper phthalocyanine tetrasulfonic acid (CuTSPc; Fig. 1 displays its molecular structure) was first applied to establish a novel assay of proteins based on the measurement of enhanced RLS. CuTSPc displays weak RLS; when trace amounts of proteins are added to its

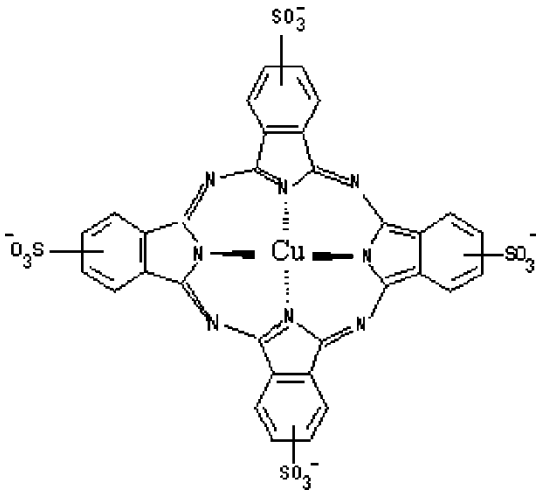

Figure 1. Molecular structure of CuTSPc.

aqueous solution, the interaction of CuTSPc with proteins results in strong enhanced RLS signals. With enhanced RLS, proteins can be determined with high sensitivity. In addition, this assay exhibits a promising improvement in simplicity, because CuTSPc is easily synthesized and chemically stable compared with most organic dyes, making this method suitable for practical applications. We apply this method to the determination of proteins in human serum samples and the results are satisfactory.

EXPERIMENTAL

Chemicals

Human serum albumin (HSA), bovine serum albumin (BSA), ovalbumin and human γ-IgG were purchased from Sigma Company (St. Louis, MO, USA) and dissolved in water to give a suitable stock solution. These stocks needed to be stored at 0–4°C and only occasionally gently shaken if needed.

A stock solution of CuTSPc (Acros Organics Company, Geel, Belgium) was prepared by dissolving 0.0982 g CuTSPc in water then diluting to 100 mL with doubly deionized distilled water at a concentration of 1.0×10^{-3} mol/L. A working solution of CuTSPc was prepared by diluting the stock solution to a concentration of 1.0×10^{-4} mol/L. Both the stock and working solutions can be stored at room temperature. It was observed that the CuTSPc solution was very stable; the stock solution could be used after 1 year of storage.

NaAc-HCl buffer solution (pH 2.60) was used to control the acidity, while a 0.5 mol/L NaCl solution was used to adjust the ionic strength of the aqueous solution.

All reagents were of analytical grade and used without further purification. The water used was doubly distilled.

Apparatus

The RLS spectrum and the intensity of RLS were measured with a F-2500 mode spectrofluorimeter (Hitachi, Tokyo, Japan) by using a 1.00-cm quartz fluorescence cell. All absorption spectra were measured on U-3010 mode spectrophotometer (Hitachi). All pH measurements were made with a pH-3C pH meter (Shanghai Leici Company, Shanghai, China).

Procedures

Into a 15-mL volumetric flask were added an appropriate working solution of proteins, 1.5 mL of a pH 2.60 buffer solution, and 3.0 mL CuTSPc (1.0×10^{-5} mol/L) solution. The mixture was finally diluted with doubly

distilled water to 15 mL and thoroughly mixed. The RLS spectrum was recorded by scanning simultaneously the excitation and emission monochromators ($\Delta\lambda = 0$ nm) of the F-2500 fluorescence spectrophotometer from 220 to 650 nm. The RLS intensity was measured at 382 nm.

RESULTS AND DISCUSSION

Spectral Characteristics

Lines 1 and 2 in Fig. 2 display the RLS features of HSA and CuTSPc, respectively. It can be seen that the RLS signal of CuTSPc is stronger than that of HSA in the whole scanning region. However, the RLS intensity of both HSA and CuTSPc are weaker than that of their mixtures over the wavelength range 220–650 nm. It was found that the RLS signals of the mixture increase with increasing HSA concentration but change slightly with the CuTSPc concentration. Namely, the RLS signal of CuTSPc is enhanced in the presence of HSA.

Figure 3 gives the absorption spectrum of CuTSPc in the presence of HSA. From the absorption spectrum of CuTSPc, it can be seen that CuTSPc has two absorption peaks at 612.7 nm and 688.2 nm, respectively, which is the Q absorption band of CuTSPc. Also, a strong shoulder at 260–450 nm can be observed, which is the Soret absorption band of CuTSPc. When HSA was added to CuTSPc solution, the absorption spectra changed

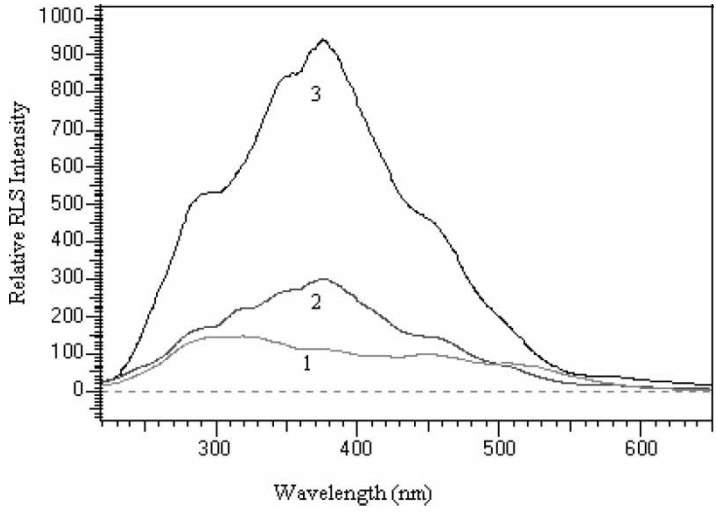

Figure 2. RLS spectra of CuTSPc and HSA. 1, HSA; 2, CuTSPc; 3, HSA-CuTSPc. Concentrations: CuTSPc, 2.0×10^{-6} mol/L; HSA, 1.0 μg/mL; pH 2.60; ionic strength 0.02 mol/L (NaCl).

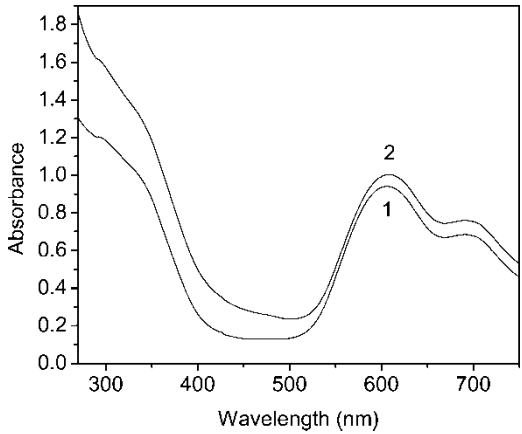

Figure 3. Absorption spectra of CuTSPc in the absence and presence of HSA. 1, CuTSPc; 2, HSA-CuTSPc. [CuTSPc]: 2.0×10^{-6} mol/L; [HSA]: 1.0 μg/mL; pH 2.60; ionic strength 0.02 mol/L (NaCl).

slightly, while the RLS spectra changed a lot (as Fig. 2 shows), which indicates an interaction between CuTSPc and proteins. Here, it can be seen that the peak of light scattering of the complex does not appear within the window of the absorption spectrum of it, so the reaction system could be treated as transparent solution. Therefore, the quantitative basis for this assay is in accordance with the Rayleigh formula:[21]

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

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_θ is the wavelength, ν is the size of the scattering particles, and N_0 is the number of particles per unit. When the species of the system is fixed, then ν is constant. Because the scattering particle is an electrostatic CuTSPc–protein complex, the number of scattering particles are determined by the concentration of protein added at a fixed concentration of CuTSPc–protein. Therefore, N_0 is proportional to the concentration of the protein added, that is, at fixed experimental conditions, the scattering light intensity I is only determined by the protein concentration C ($I = K_c$).

Optimization of the General Procedure

Effect of pH

The effect of pH on the RLS signal of the assay system was studied. As shown in Fig. 4, the RLS intensities display different tendencies depending on the pH

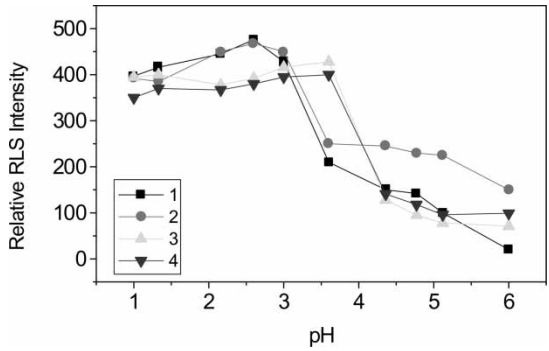

Figure 4. Effect of pH on relative RLS intensity. 1, HSA; 2, BSA; 3, human γ-IgG; 4, ovalbumin. [CuTSPc]: 2.0×10^{-6} mol/L; [protein]: 1.0 μg/mL; ionic strength 0.02 mol/L (NaCl).

values of the medium. With increasing pH value of the medium, the RLS intensity of CuTSPc mixed with protein displays different features. CuTSPc in the presence of HSA or BSA has the maximum RLS response in the range of pH 2–3, and in the presence of human γ-IgG or ovalbumin has its maximum RLS response in the range of 3–4. Proteins are negatively charged if the pH of the medium is higher than their isoelectric points (pI). Thus, the pH dependence shown in Fig. 4 possibly displays electrostatic attraction between CuTSPc with proteins.

Optimum Amounts of CuTSPc

The effect of CuTSPc concentration on the RLS signal of the assay system is shown in Fig. 5. Maximum enhancement occurred when the concentration of CuTSPc was varied between 1.0×10^{-6} and 8.0×10^{-6} mol/L. Therefore, in our experiment, 3.0 mL of 1.0×10^{-5} mol/L CuTSPc was applied.

Incubation Time and Stability

In some protein assays, enough incubation time is needed before detection.[22,23] However, the relative RLS intensity in this system reached the maximum right after the reagents were mixed. No additional incubation time is needed in this assay. Further research proved that I_{RLS} in this system kept constant for about 2 hr. Therefore, this procedure is simple and rapid with good stability. All the RLS was measured after the reagents were mixed together.

The Effect of Ionic Strength

The interaction of proteins and CuTSPc is mainly a result of electrostatic forces, so the ionic strength should have some effects on the reaction. It has

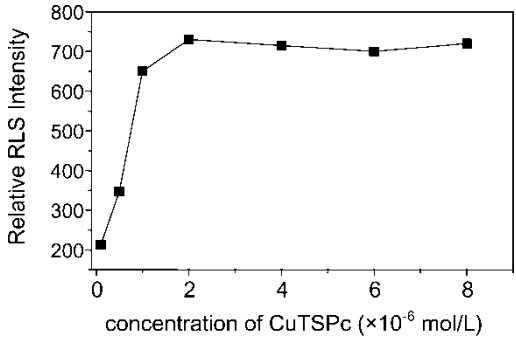

Figure 5. Effect of different concentration of CuTSPc. [HSA]: 1.0 μg/mL; pH 2.60; ionic strength 0.02 mol/L (NaCl).

been found that the intensity of RLS is stable (as shown in Fig. 6) 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 a decrease of the electrostatic forces interaction between the CuTSPc and proteins, because of the shielding effect of the charges on proteins with increasing ionic strength, which is not beneficial to the formation of the CuTSPc–proteins complex.

Interfering Substance

Tolerance of foreign substances is of importance for the analytical application of a proposed method. The effects of substances including common ions and amino acids were examined for interference. The results are summarized in Table 1. Table 1 shows that most of the metal ions in an organism, such as K^+, Ca^{2+}, and Mg^{2+}, can be allowed at relative high concentrations,

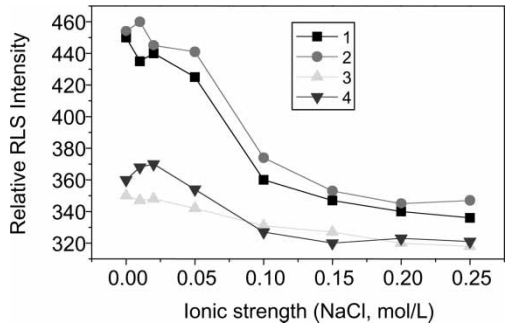

Figure 6. Dependence of relative RLS intensity on ionic strength. 1, HSA; 2, BSA; 3, human γ-IgG; 4, ovalbumin. [CuTSPc]: 2.0×10^{-6} mol/L; [protein]: 1.0 μg/mL; pH, 2.60.

Table 1. Effect of foreign substances on the determination of human serum albumin (1.0 μg/mL) under the optimum conditions

No.	Substance	Concentration	Change of I_{RLS} (%)	No.	Substance	Concentration	Change of I_{RLS} (%)
1	K^+, Cl^-	0.1 mmol/L	−3.7	11	L- Try	100 μg/mL	−3.2
2	Ca^{2+}, Cl^-	0.1 mmol/L	−3.2	12	L- Cys	100 μg/mL	−3.0
3	Mg^{2+}, Cl^-	0.1 mmol/L	+4.1	13	L- Phe	100 μg/mL	−2.7
4	Co^{2+}, Cl^-	0.05 mmol/L	−4.8	14	L- Tyr	100 μg/mL	−3.5
5	Ni^{2+}, Cl^-	0.05 mmol/L	+3.9	15	L- Arg	100 μg/mL	−1.5
6	Zn^{2+}, Cl^-	0.05 mmol/L	+4.7	16	L- Leu	100 μg/mL	−1.8
7	Cr^{3+}, Cl^-	0.1 μg/mL	+6.7	17	L- Gly	100 μg/mL	−2.7
8	Pb^{2+}, NO_3^-	0.1 μg/mL	+8.9	18	L- His	100 μg/mL	−1.6
9	Hg^{2+}, Cl^-	0.1 μg/mL	+7.2	19	L- Pro	100 μg/mL	−3.1
10	Cu^{2+}, Cl^-	0.1 μg/mL	+5.6	20	L- Asp	100 μg/mL	−2.4

whereas such ions as Hg^{2+}, Cr^{3+}, Co^{2+}, and Cu^{2+} can be allowed only at very low concentrations. However, these ions in real samples, such as human serum, can be minimized by diluting with water. In addition, this assay is not interfered with by amino acids.

Calibration Curves

According to the general procedures, the relationships between the RLS intensity and the protein concentration were obtained. The results are listed in Table 2. It can be seen that for different proteins, the sensitivity or the slope of the linear regression was different. It was been reported[24–26] that the enhanced RLS signals appear to depend sensitively on the electric properties of the individual chromophores, the strength of the electrostatic interaction between the chromophores and proteins, and the size of the formed complex. Because the size and electric charges of an individual protein vary with the kind of protein, it can be concluded that the RLS responses are dependent on protein variability.

Sample Analyses

Calibration curves for HSA and human γ-IgG have different slopes. In order to determine the total protein in human serum samples, the calibration curve for standard human serum has to be established first. The standard human serum was obtained by mixing 40 normal human serum samples, followed by the determination of its total content of proteins using the Bradford method.[2] The construction of a calibration curve and the determination of protein in serum samples were performed according to the procedures described above. Human serum samples were obtained from three different donors of the hospital of Anhui Normal University and diluted 10,000-fold with doubly distilled water, and the determination results are listed in Table 3. By comparing the results of the current method with the values given by the hospital, it is clear that the determination for the total content of proteins is reliable, sensitive, and practical.

Table 2. Analytical parameters for proteins by the RLS method

Proteins	Linear regression equation (C: μg/mL)	Linear range (μg/mL)	Detection limit (ng/mL)	Correlation coefficient (r)
HSA	$\Delta I_{RLS} = -1.2 + 23.6C$	0.1–2.0	23.4	0.9923
BSA	$\Delta I_{RLS} = -0.7 + 19.8C$	0.06–4.0	16.8	0.9916
γ-IgG	$\Delta I_{RLS} = -0.1 + 11.4C$	0.0–2.0	37.6	0.9908
Ovalbumin	$\Delta I_{RLS} = -0.4 + 12.7C$	0.2–6.0	48.3	0.9954

HSA, human serum albumin; BSA, bovine serum albumin.

Table 3. Analytical Results of human serum samples by the RLS method

Samples (No.)	This method (mg/mL, n = 5)	Clinical data (mg/mL)
1	77.2 ± 2.2	76.3
2	73.4 ± 2.9	72.9
3	70.0 ± 3.4	69.1

CONCLUSIONS

It is found that the interaction of CuTSPc with proteins resulted in the enhancement of RLS intensity of CuTSPc, proposing a novel method for determination of four kinds of proteins. Besides its high tolerance of accuracy and simplicity, and high sensitivity, it has some other advantages: rapidity of reaction (no incubation time), stability of complex formed, and reproducibility. Moreover, compared with other dyes, CuTSPc is very stable, relatively inexpensive, and easily synthetic. Therefore, this method has potential to be expanded to applications in biochemistry and clinic practice.

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