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Determination of Nanograms of Nucleic Acids by Their Enhancement Effect on the Resonance Light Scattering of the Cobalt(II)/ 4-[(5-Chloro-2-pyridyl)azo]-1,3-diaminobenzene Complex

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Using a common spectrofluorometer to measure the intensity of resonance light-scattering, a method for determination of nucleic acids in the nanogram range has been developed. In the pH range 11.5−12.0, the resonance light-scattering of the binary comlpex of cobalt(II)/ 4-[(5-chloro-2-pyridyl)azo]-1,3-diaminobenzene (5-Cl-PADAB) is greatly enhanced by nucleic acids, with the maximum scattering peak located at 547.0 nm. The enhanced intensity of resonance light-scattering is in proportion to the concentration of calf thymus DNA in the range 0−400 ng/mL and to that of fish sperm DNA and yeast RNA in the range 0−300 ng/mL. The limits of detection are 1.4 ng/mL for calf thymus DNA, 0.8 ng/ mL for fish sperm DNA, and 1.3 ng/mL for yeast RNA. Precision at 200 ng/mL for the three nucleic acids is 1.9%, 2.0%, and 0.8%, respectively. Six synthetic samples were determined satisfactorily. Mechanism studies showed that the nature of the reaction is that the binary complex of Co(II)/5-Cl-PADAB reacts with single-stranded nucleic acid, and the enhancement effect of nucleic acids on the resonance light scattering of the binary complex is due to the stacking of the binary complex on nucleic acids, which act as a template.

The quantitative analysis of nucleic acids is very important because it can be used as a reference for measurements of other components in biological fluids and genetic diagnosis. The most sensitive quantitation of nucleic acids at present is generally according to their fluorescence enhancement effect on organic dyes such as ethidium bromide (EB) and its homo- or heterodimer,[1,2] diaminophenylindole (DAPI),[3] and bisimidazole (Hoechst 33258).[4] By using laser facilities and the fluorescence enhancement effect of nucleic acids on the organic dyes with double functional groups, single DNA molecules and the growth of DNA in PCR can be detected.[5,6]

Scattering light is highly applicable to the polymer sciences, and particularly dynamic Rayleigh scattering is an important tool for the study of translational and rotational motions of molecules in solutions.[7] The technique, however, suffers the disadvantages of low signal levels and lack of sensitivity unless laser facilities are employed. Recently, Pasternack et al.[8−11] developed a technique to detect the intensity of scattering light by using a common spectrofluorometer. We think the technique is useful in analytical chemistry because of the simplicity and sensitivty. With this technique, we have established a sensitive method for studying trace amounts of biological substances.[12] In this paper, we present a new method for determination of nucleic acids with high sensitivity and discuss the reaction mechanism according to the resonance light-scattering data. The basis for the method is the enhancement effect of nucleic acids on the scattering light of the binary complex of Co(II)/4-[(5-chloro-2-pyridyl)azo]-1,3-diaminobenzene (5-Cl-PADAB).

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(1) (a) Pandey, P. C.; Weetall, H. H. *Anal. Chem*. **1995**, *67*, 787−792. (b) Harriman, W. O.; Wabl, M. *Anal. Biochem*. **1995**, *228*, 336−342. (c) Strothkamp, K. G.; Strothkamp, R. E. *J. Chem. Educ*. **1994**, *71*, 77−79. (d) Sari, M. A.; Battioni, J. P.; Duppre, D.; Mansuy, D.; Le Peck, J. B. *Biochemistry* **1990**, *29*, 4205−4215. (e) Markovits, J.; Roques, B. P.; Le Pecq, J. B. *Anal. Biochem*. **1979**, *94*, 259−264.

(2) (a) Piuno, P. A. E.; Krull, U. J. *Anal. Chem*. **1995**, *67*, 2635−643. (b) Piuno, P. A. E.; Krull, U. J.; Hudson, R. H. E.; Damha, M. J.; Cohen, H. *Anal. Chim. Acta* **1994**, *288*, 205−214.

(3) (a) Daxhelet, G. A.; Kohnen, M. M.; Coene, M. M.; Hoet, P. P. *Anal. Biochem*. **1990**, *190*, 116−119. (b) Daxhelet, G. A.; Coene, M. M.; Hoet, P. P.; Cocito, C. G. *Anal. Biochem*. **1989**, *179*, 401−403.

(4) (a) Rao, J.; Otto, W. R. *Anal. Biochem*. **1992**, *207*, 186−192. (b) Rago, R.; Mitchen, J.; Wilding, G. *Anal. Biochem*. **1990**, *191*, 31−34. (c) Lipman, J. M. *Anal. Biochem*. **1989**, *176*, 128−131.

(5) (a) Benson, S. C.; Zeng, Z. X.; Glazer, A. N. *Anal. Biochem*. **1995**, *231*, 247−255; 256−260. (b) Rye, H. S.; Drees, B. L.; Nelson, H. C. M.; Glazer, A. N. *J. Biol. Chem*. **1993**, *268*, 25229−25238. (c) Glazer, A. N.; Rye, H. S. *Nature (London)* **1992**, *359*, 589−561. (d) Petty, J. T.; Johnson, M. E.; Goodwin, P. M.; Martin, J. C.; Jett, J. H.; Keller, R. A. *Anal. Chem*. **1995**, *67*, 1755−1761. (e) Haab, B. B.; Mathies, R. A. *Anal. Chem*. **1995**, *67*, 3253−3260. (f) Zhu, H. P.; Clark, S. M.; Benson, S. C.; Rye, H. S.; Glazer, A. N.; Mathies, R. A. *Anal. Chem*. **1994**, *66*, 1941−1948.

(6) (a) Skogerboe, K. J. *Anal. Chem*. **1995**, *67*, 499R−4554R. (b) Perez-Howard, G. M.; Weil, P. A.; Beenchem, J. M. *Biochemistry* **1995**, *34*, 8005−8017. (c) Devlin, R.; Studholme, R. M.; Dandliker, W. B.; Fahy, E.; Blumeyer, K.; Ghosh, S. S. *Clin. Chem*. **1993**, *65*, 2352−2359.

(7) Zuo, J. *The Principles and Applications of Laser Light Scattering in Polymer Science*; Henan Science and Technology Press: Zhengzhou, 1994; pp 1−180.

(8) Pasternack, R. F.; Collings, P. J. *Science* **1995**, *269*, 935−939.

(9) Pasternack, R. F.; Bustamante, C.; Collings, P. J.; Giannetto, A.; Gibbs, E. J. *J. Am. Chem. Soc*. **1993**, *115*, 5393−5399.

(10) (a) Paula, J. C.; Robblee, J. H.; Pasternack, R. F. *Biophys. J*. **1995**, *68*, 335−341. (b) Pasternack, R. F.; Schaefer, K. F. *Inorg. Chem*. **1994**, 433, 2062−2065.

(11) Arena, G.; Scolaro, L. M.; Pasternack, R. F.; Romeo, R. *Inorg. Chem*. **1995**, *34*, 2994−3002.

(12) Huang, C. Z.; Li, K. A.; Tong, S. Y. *Anal. Chem*. **1996**, *68*, 2259−2263.

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The intensity of Rayleigh light scattering by transparent isotropic media is in proportion to λ^{-4}, where λ is the wavelength of incident light in free space. The intensity deviates from the dependence of λ^{-4}, and it is possible that the intensity become quite large when the incident wavelength is near the absorption band of the analyte molecules. This phenomenon is known as resonance-enhanced Rayleigh scattering and is predicted by the same theory that predicts resonance-enhanced Raman scattering.[13] According to macroscopic fluctuation theory and Mie theory, Miller[14] had made theoretical studies. Further theoretical and practical studies had been made by Anglister and Steinberg,[15] Stanton and Pecora,[13] and Pasternack et al.[8−11] In general, the intensity of light scattering depends on the volume of the species, the wavelength of incident light, and the real and imaginary parts of the scatterer's polarizability. So strong light-scattering bands are expected for large aggregates at the wavelength where the molar absorptivity of the aggregate is large, but very weak or no light-scattering signals can be detected for those species whose volume are small, even if the incident wavelength is close to their strong absorption bands. With resonance light-scattering spectroscopy, the self-aggregation of chlorophyll α and sulfotophenylporphyrins[10] and the interaction of the metallointercalator cationic complex (2,2′:6′,2″-terpyridine)methylplatinum(II) with DNA[11] have been studied.

5-Cl-PADAB is a commercial organic reagent and has extensive applications in the sensitive determination of metal ions.[16] As we previously reported,[17] the interaction of the binary complex of Co(III)/5-PADAB with nucleic acids occurs in such a way that each nucleotide residue can bind two molecules of the binary complex. That mimics the stacking of the binary complex on nucleic acids, which act as a template.[18] Since the stacking species have high absorptivity (for example, ϵ(ctDNA/Co(III)/5-Cl-PADAB) $= 5.1 \times 10^4$ M^{-1} cm^{-1} [19]) and possibly large volume, it is expected that the resonance light scattering of the binary complex can be enhanced by nucleic acids.

EXPERIMENTAL SECTION

Apparatus. The resonance light-scattering spectrum and the intensity of resonance light scattering were measured with a Shimadzu RF-540 spectrofluorometer (Kyoto, Japan). A WH-861 vortex mixer (Huangjin Instrumental Co., Jiangsu, China) was used to blend the solution.

Reagents. Stock solutions of nucleic acids were prepared by directly dissolving commercial calf thymus DNA (Beitai Biochemical Co., Chinese Academy of Sciences, Beijing, China), fish sperm DNA, and yeast RNA (Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai, China) in doubly deionized water at 0−4 °C. Twenty-four hours or more was needed for complete dissolution of DNAs, even if occasional gentle shaking was done. The concentrations of DNAs were calculated according to the absorbance at 260.0 nm. All the working concentrations of nucleic acids were 2.5 μg/mL.

The stock solution of Co(II) was prepared by dissolving cobalt metal (99.99%) in nitric acid, and the working solution of Co(II) was obtained by diluting the stock solution to 1.0×10^{-4} M with water. The stock solution of 5-Cl-PADAB was prepared by dissolving 61.92 mg of the crystallized 5-Cl-PADAB (Merck, Germany) in thermal dehydrated alcohol, and after the solution had been cooled, dehydrated alcohol was added to 500 mL. The working solution of 5-Cl-PADAB was made 1.0×10^{-4} M (containing 40% (v/v) ethanol) by diluting the stock solution with water. In addition, 0.01 and 0.30 M NaOH solutions were used.

All reagents were of analytical grade without further purification. Water used throughout was doubly deionized.

Preparation of Synthetic Samples. According to the tolerances of foreign substances, interfering components were added in an appropriate volume of standard solution to make up synthetic samples. To test the practicability of the method, six samples were constructed.

Standard Procedure. In a dry 10-mL volumetric flask were added 0.50 mL of Co(II) solution, 0.50 mL of 5-Cl-PADAB solution, 0.25 mL of dehydrated alcohol, and 1.0 mL of 0.3 M NaOH. The mixture was vortexed, and then nucleic acid standard solution or sample solution was added, and the mixture was vortexed again. Before the addition of nucleic acids, it was necessary to add an appropriate volume of water to keep the total initial volume of the mixture at 3.40 mL. Fifteen minutes later, the mixture was diluted to 10 mL with doubly deionized water and mixed thoroughly. The resonance light-scattering spectrum and the intensity of scattering were measured against the binary complex as a reference during the period of 20−100 min after the last mix.

The resonance light-scattering spectrum was obtained by scanning simultaneously the excitation and emission monochromators of the RF-540 spectrofluorometer from 400 to 700 nm (namely, $\Delta\lambda = 0$ nm). The intensity of light scattering was measured at the wavelength where the maximum scattering peak is located. Both the intensity measurement and the spectrum scanning of the resonance light scattering were made by keeping the slit-width of the excitation and the emission of the spectrofluorometer at 5.0 nm.

RESULTS AND DISCUSSION

Spectral Characteristics. Figure 1 displays the resonance light-scattering spectrum of the binary complex of Co(II)/5-Cl-PADAB and its enhanced resonance light-scattering spectra by nucleic acids. In the wavelength range 400−650 nm, the resonance light scattering of the binary complex is rather weak; even so, the resonance light scattering in the 540−600 nm wavelength range is stronger than that in the 480−510 nm range because of the molecular absorption of the binary complex (see below). In the wavelength range 400−700 nm, the resonance light scattering of the binary complex is enhanced by nucleic acids, with the maximum scattering peak located at 547.0 nm. Shoulder peaks in the range 480−510 nm can be observed. In addition, the enhanced extent of the light scattering differs for different nucleic acids. Figure 1 shows the enhanced order for different nucleic acids as follows: fish sperm DNA > yeast RNA > calf thymus DNA (on the basis of ng/mL). As a matter of fact, the comparison for the enhanced order should be based on the molecular mass or the length of nucleic acids. By using $\epsilon_{DNA} = 6600$ M^{-1} and

(13) Stanton, S. G.; Pecora, P. *J. Phys. Chem.* **1981**, *75*, 5615−5626.
(14) Miller, G. A. *J. Phys. Chem.* **1978**, *82*, 616−618.
(15) (a) Anglister, J.; Steinberg, I. Z. *Chem. Phys. Lett.* **1979**, *65*, 50−54. (b) Anglister, J.; Steinberg, I. Z. *J. Chem. Phys.* **1983**, *78*, 5358−5368.
(16) Cheng, K. L.; Ueno, K.; Imamura, T. *CRC Handbook of Organic Analytical Reagents (Chinese Version)*; Geology Press: Beijing, 1982; pp 142−143.
(17) Huang, C. Z.; Li, K. A.; Tong, S. Y. *Anal. Chim. Acta*, in press.
(18) Pasternack, R. F.; Gibbs, E. J. *J. Inorg. Organomet. Polym.* **1993**, *3*, 77−88.
(19) Huang, C. Z. Ph.D. Dissertation, Peking University, Beijing, 1996; p 35.

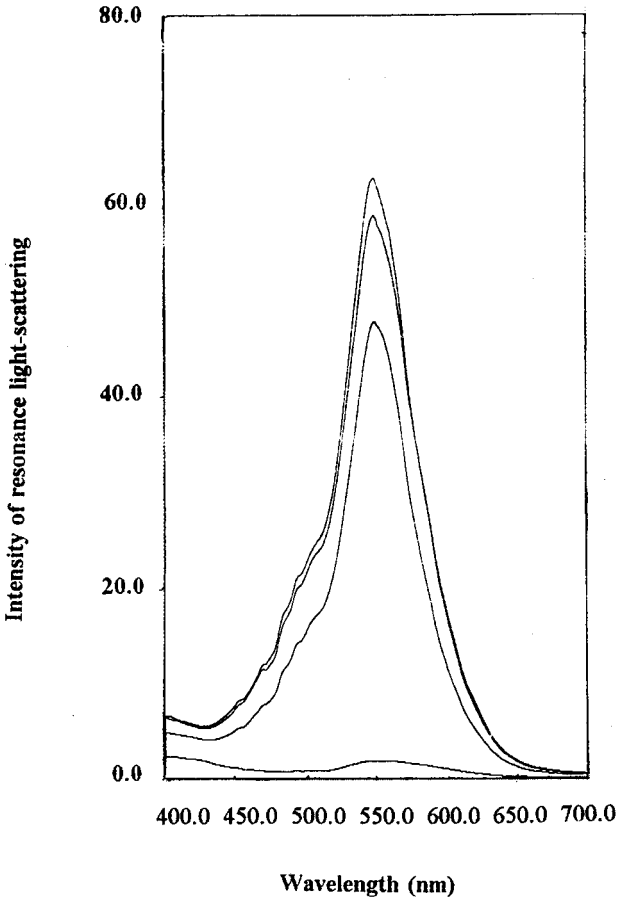

Figure 1. Resonance light-scattering spectra. From top to bottom, ternary systems of fish sperm DNA, yeast RNA, calf thymus DNA, and the binary complex of Co(II)/5-Cl-PADAB. Concentrations: calf thymus DNA, 200.0 ng/mL; $c_{Co(II)} = 0.5c_{5\text{-Cl-PADAB}} = 5.0 \times 10^{-6}$ M; ethanol, 12.9% (v/v) in period 1.

$\epsilon_{RNA} = 7800\ M^{-1}\ cm^{-1}$,[20] we find that the enhanced order yeast RNA > fish sperm DNA > calf thymus DNA (on the basis of mol/L) is followed.

The features of those spectra can be elucidated by the theory of resonance depolarized Rayleigh scattering. When the incident light passes through a transparent isotropic medium, the intensity of light scattering is proportional to λ^{-4}, but if λ is near an absorption band of the molecules and if there are aggregates in the system, the scattering cross section of the system, C_{sca}, the ratio of the rate of energy scattering out of the incident beam (in all directions) to the intensity of the incident beam, can be expressed as[8,9,13−15]

$$C_{sca} = (k_m^4)|\alpha|^2/(6\pi) = (k_m^4)(\alpha_r^2 + \alpha_i^2)/(6\pi) \qquad (1)$$

where k_m is the wave vector of light in the solvent, $k_m = 2\pi/\lambda_m$, and α_r and α_i are the real and imaginary parts of the polarizability of the aggregates. The absorption cross section of the system, C_{abs}, the ratio of the rate of energy absorption from the incident beam to the intensity of the incident beam, depends on α_i only,

$$C_{abs} = k_m\alpha_i \qquad (2)$$

and the absorbance A of a sample of thickness L is

$$A = 2.3^{-1}(N/V)C_{abs}L \qquad (3)$$

where N/V is the number of aggregates per unit volume. From the C_{abs} equation, the absorption depends on the first power of the polarizability, which in turn depends linearly on the volume of the aggregate. Thus, a solution with a fixed concentration of the aggregating component will exhibit no change in A as aggregation occurs, because the product of N/V and α_1 stays constant. However, the intensity of scattering depends on the square of the volume of the aggregate, and thus it increases as a result of aggregation; resonance light scattering is, therefore, extremely sensitive to even low concentrations of extended aggregates.

The binary complex has high absorptivity,[16] but it does not show aggregating tendency under the experimental case and has small volume compared with aggregate particles, so its resonance light scattering is rather weak. Even so, since the binary complex has an absorption band in the range 480−510 nm (Figure 2), the absorption band usually reducing the resonance light scattering for those species which have small volume, the resonance light-scattering of the binary complex in the 480−510 nm absorption region is smaller than that in the range 540−600 nm (Figure 1), where the binary complex has no absorption band. When the binary complex interacts with nucleic acids, enhanced resonance light-scattering spectra can be obtained (Figure 2). Compared with the resonance light-scattering spectrum of the binary complex, the enhanced resonance light-scattering spectra have strong resonance light scattering, with the maximum scattering peak located at 547.0 nm (Figure 1). The maximum scattering peak is almost the same as the maximum absorption wavelength of the ternary system near 545.0 nm (Figure 2). As we previously reported,[17] the interaction mechanism of the binary complex with nucleic acids mimics the stacking of the binary complex on the nucleic acids, which act as a template, so the enhanced resonance light scattering, with its maximum scattering peak located at 547.0 nm, results from the enhancement effect of Rayleigh light-scattering of the binary complex by nucleic acids. The shoulder peaks, observed in the range 480−510 nm, which may disclose the absorption of the binary complex in the ternary system, support the stacking mechanism of the binary complex on nucleic acids.

Optimization of the General Procedure. By using 200.0 ng/mL calf thymus DNA, 5.0×10^{-6} M Co(II), and 1.0×10^{-5} M 5-Cl-PADAB (the choice of the concentration of Co(II) and 5-Cl-PADAB will be explained later), the optimal conditions were tested. To understand the reaction better, we divided the reaction into two periods, one before the dilution to 10 mL and the other after the dilution. Figure 3 shows that the maximum light scattering takes place in the pH range 11.5−12.0 in period 1.

Ionic strength and the content of ethanol have significant effects on the interaction. From Figure 4, it can be seen that, when ionic strength is low ($I < 0.1$), the reaction is scarcely affected. However, with the increase of ionic strength, the reaction slows down. If the ionic strength is too large, the interaction is blocked. Figure 5 shows the effect of ethanol. When the content

(20) Chen, Z.; Liu, J.; Luo, D. *Biochemistry Experiments*; Chinese University of Sciences and Technology Press: Hefei, PRC, 1994; p 111.

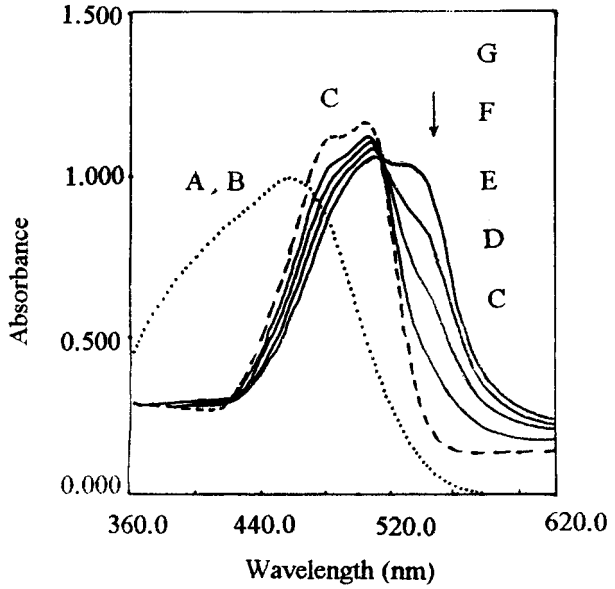

Figure 2. Absorption spectra of 5-Cl-PADAB(A), DNA + 5-Cl-PADAB(B), Co(II) + 5-Cl-PADAB(C), and Co(II) + 5-Cl-PADAB + DNA(D-G). Concentrations: $c_{Co(II)} = 0.5c_{5\text{-Cl-PADAB}} = 2.0 \times 10^{-5}$ M; calf thymus DNA, D 1.0, E 2.0, F 3.0, G 4.0 μg/mL; ethanol, 20.7% (v/v) in period 1.

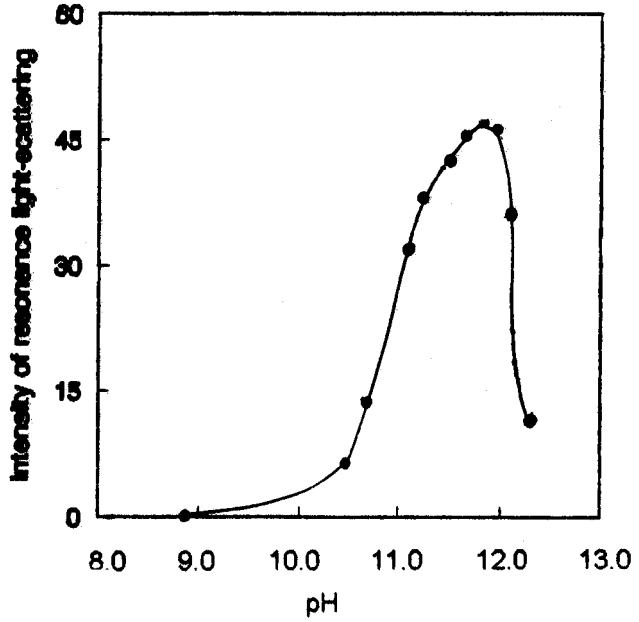

Figure 3. Dependence of the enhancement of RLS on pH. Concentrations: calf thymus DNA, 200 ng/mL; $c_{Co(II)} = 0.5c_{5\text{-Cl-PADAB}} = 5.0 \times 10^{-6}$ M; ethanol, 12.9% (v/v).

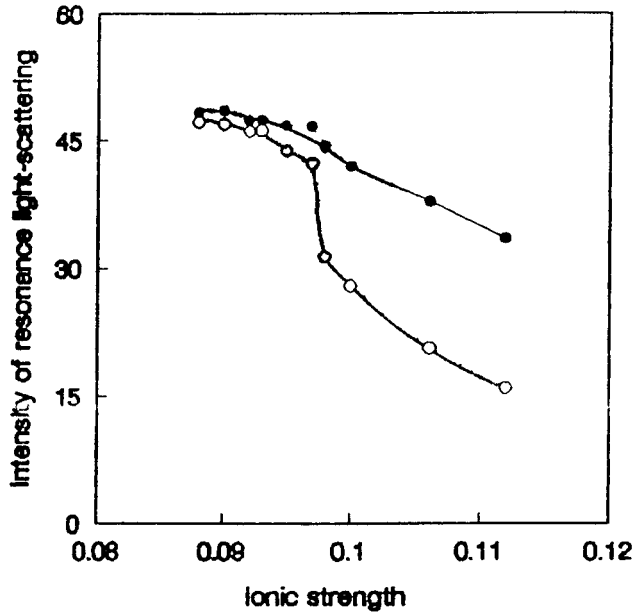

Figure 4. Influence of ionic strength on the enhancement of RLS. Intensity of resonance of light-scattering was obtained at 20 (○) and 60 min (●). Calf thymus DNA, 200.0 ng/mL; $c_{Co(II)} = 0.5c_{5\text{-Cl-PADAB}} = 5.0 \times 10^{-6}$ M; ethanol, 12.9% (v/v).

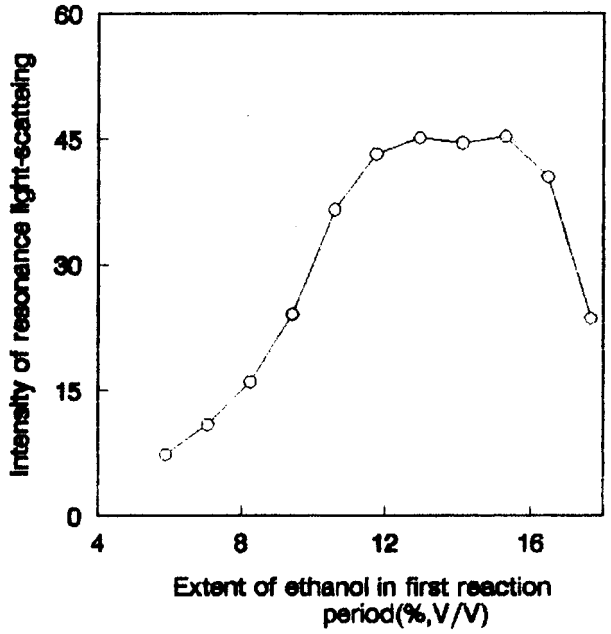

Figure 5. Effect of the content of ethanol. Concentrations: calf thymus DNA, 200.0 ng/mL; $c_{CO(II)} = 0.5c_{5\text{-Cl-PADAB}} = 5.0 \times 10^{-6}$ M.

of ethanol is low, the scattering is weak and stable, but with the increase of the content of ethanol, the scattering becomes strong and keeps constant when the content of ethanol is in the range 11.8−15.3% (v/v). If the content of ethanol is too large, the reaction is blocked. The effects of both ionic strength and ethanol content on the interaction might result from the conformation variation of nucleic acids. With increasing ionic strength, the anion of phosphate on the backbone is shielded by the cation ion of the ionic strength controller (Na^+, for instance), and the separation of the double strands becomes very difficult,[21] i.e., unfavorable to the stacking of the binary complex on the single-stranded nucleic acids. Similarly, with increasing the content of ethanol, variation of the conformation of nucleic acids will take place,[21] so it is difficult for the binary complex to stack on the single-stranded nucleic acids.

Figure 6 depicts the influence of the volume of the mixture in the first reaction period. It shows that, the larger the volume of the solution mixture in period 1, the smaller the intensity of resonance light scattering is. So, the division of the reaction into two periods is of benefit to the sensitivity. According to Figure 6, we can calculate the optimal content of ethanol ranging from 14.8 to 11.7% (v/v), which parallels the finding of Figure 5. In addition, we found that the reaction time of period 1 plays a very important role, and the suitable reaction time of period 1 is 12−20 min. Since alkaline denaturation of nucleic acids should take

(21) Sun, L. E.; Sun, D. X.; Zhu, D. X. *Molecular Genetics*; Nanjing University Press: Nanjing, 1995; pp 6−28.

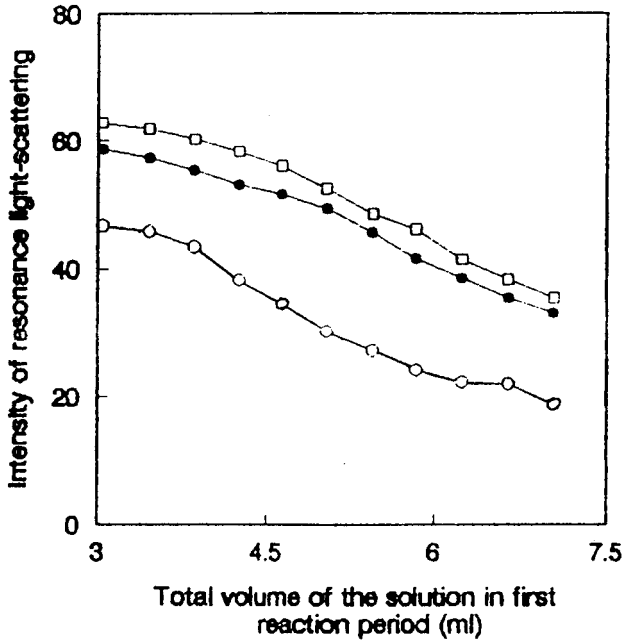

Figure 6. Choice of the volume of the mixture in period 1. The total volume of ethanol coming from 5-Cl-PADAB and extra addition is 0.45 mL, and the content of ethanol can be calculated from the total volume of ethanol (0.45 mL) and the total volume of the mixture in the period 1. Concentration of nucleic acids, 200.0 ng/mL; $c_{Co(II)} = 0.5c_{5\text{-Cl-PADAB}} = 5.0 \times 10^{-6}$ M. From top to bottom, fish sperm DNA, yeast RNA, and calf thymus DNA.

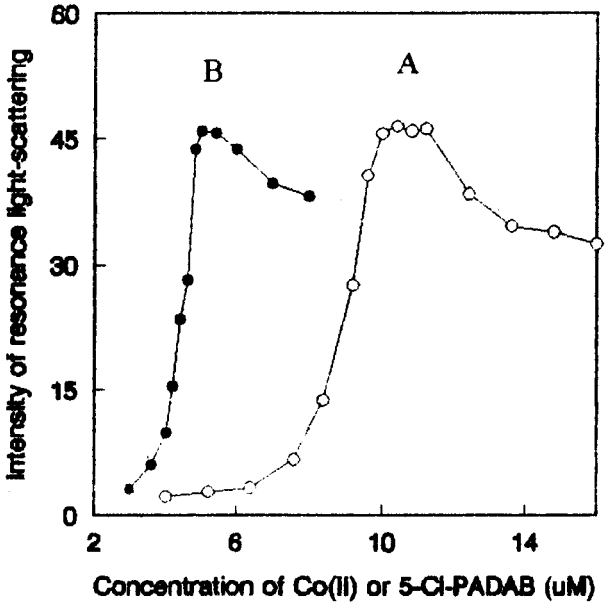

Figure 7. Test of molar ratio of Co(II)/5-Cl-PADAB upon fixing the concentration of Co(II) at 5.0×10^{-6} M (A) or 5-Cl-PADAB at 1.0×10^{-5} M (B), with the concentration of another component changing. Concentration of calf thymus DNA was 200.0 ng/mL, and the content of ethanol was kept at 12.9% (v/v).

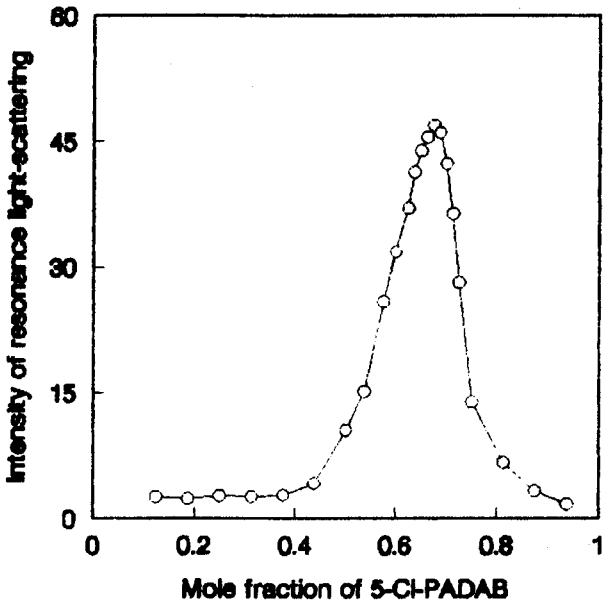

Figure 8. Test of molar ratio of Co(II)/5-Cl-PADAB upon keeping the total concentration of Co(II) and 5-Cl-PADAB at 1.6×10^{-5} M with the concentrations of the two components changing simultaneously. Concentrations of DNA and content of ethanol are the same as in Figure 5.

place at pH > 11.3,[21] it is possible that, in period 1, the interaction of the binary complex with nucleic acids involves the separation of the double strands of nucleic acids. The use of thermally and alkalinously denatured nucleic acids to speed up the interaction[17] supports the interaction process. As for period 2, the intensity of scattering can reach its maximum in 20 min after the last dilution, and there would be a slight increase, but it is admittable with the allowed intensity error of 5% if the determination is finished in 100 min.

Molar Ratio of Co(II)/5-Cl-PADAB in the Ternary System. By spectrophotometry,[17] we have obtained that the molar ratio of Co(II) to 5-Cl-PADAB of the ternary system is 1:2, which is in agreement with the molar ratio of Co(II) to 5-Cl-PADAB of the binary complex.[16] The identical result can be obtained with the resonance light-scattering method also.

Under the experimental conditions, by keeping the concentration of nucleic acids constant, the molar ratio of Co(II) to 5-Cl-PADAB of the ternary system can be measured by two methods. One method is that the concentration of one of the two components (Co(II) and 5-Cl-PADAB) is kept constant and that of the other component is changed. Figure 7 shows that, whether the concentration of Co(II) or 5-Cl-PADAB changes with the other component being kept constant, the molar ratio of Co(II) to 5-Cl-PADAB is 1:2. The other method is to hold the total concentration of Co(II) and 5-Cl-PADAB at 1.6×10^{-5} M while the concentrations of the two components are changed simultaneously. Figure 8 shows that, when the molar fraction of 5-Cl-PADAB is 0.675, namely the molar ratio of Co(II) to 5-Cl-PADAB is 1:2, the intensity of light-scattering reaches maximum.

Nature of the Reaction. According to Figure 2, which has an isobestic point at 492.0 nm, and the molar ratio of Co(II) to 5-Cl-PADAB of the ternary system, it is apparent that the formation of the ternary system is based on the interaction of the binary complex of Co(II)/5-Cl-PADAB with nucleic acids, and maybe ternary compounds have formed. As the reaction takes place in the pH range 11.5−12.0, at the time alkaline denaturation of nucleic acids has occurred, and both the alkaline and thermal denaturation of DNA speeds up the reaction,[17] the nature of the interaction is based on the binary complex and the single-stranded nucleic acids. In addition, by spectrophotometry we found that each nucleotide residue can bind two binary complex molecules,[17] so the enhancement effect of nucleic acids on the resonance light-scattering of the binary complex is due to the stacking of binary complex on nucleic acids, which act as a template. Therefore, when the ionic strength is large, the double strands of nucleic

Table 1. Interferences of Foreign Coexisting Substances

substance	concn coexisting ($\times 10^{-6}$ M)	change of intensity of resonance light scattering (%)
protein, BSA	240.0*	6.4
protein, HSA	240.0*	9.0
protein, γ-IgG	240.0*	7.4
base, A	18.0	−7.7
base, G	0.36	−3.4
base, C	18.0	−8.7
base, T	5.0	−2.4
base, U	15.0	−2.4
PO_4^{3-}Na(I)	18.0	−7.4
Al(III), SO_4^{2-}	1.0	−9.8
Ca(II), Cl^-	3.6	−10.0
Cd(II), SO_4^{2-}	0.6	−5.6
Cr(III), Cl^-	1.8	−0.1
Cu(II), Cl^-	1.8	−7.7
Hg(II), NO_3^-	0.04	−0.2
Mg(II), Cl^-	1.0	−9.5
Mn(II), NO_3^-	0.5	−6.8
Ni(II), NO_3^-	0.5	−8.1
Pb(II), NO_3^-	1.8	−2.3
Zn(II), Cl^-	3.6	−5.0

[a] Concentrations of proteins are represented as ng/mL. All the values obtained in the table were obtained according to the standard procedure, and the concentrations of reagents are as follows: calf thymus DNA, 200.0 ng/mL; Co(II), 5.0×10^{-6} M; 5-Cl-PADAB, 10.0×10^{-6} M; ethanol (first reaction period), 12.9% (v/v).

acids are difficult to separate because of the shielding effect of Na^+, and the reaction is blocked.

Interferences of Coexisting Foreign Substances. The influences of foreign coexisting substances such as proteins, bases, and metal ions were tested. The results are presented in Table 1. Of the tested metal ions, Ca(II), Cr(III), Cu(II), Mg(II), Pb(III), and Zn(II) ions can be allowed with relatively higher concentration (the allowed maximum concentration can reach 1.8×10^{-6} M), but Hg(II) ion can be allowed with very low concentration ($<4.0 \times 10^{-8}$ mol/L). However, the allowed concentration of those interference ions is still larger than that in biological fluids.

Characteristics of the Resonances Light-Scattering Method. As stated above, resonance light-scattering spectra were obtained by scanning simultaneously the excitation and emission monochromators. So, resonance light-scattering spectra are synchronous ones. In addition, scattering light stems from the aggregate when it is excited by a light beam with the emission in all directions, and the emission wavelength is equal to the scattering light wavelength, which means the aggregate is a new luminophor that remits light with the same wavelength as the incident light. Thus, the resonance light scattering belongs to synchronous luminescence, with its particularities of $\Delta\lambda = 0$ nm and no electronic excitation after light absorption. According to the equation of synchronous luminescence,[22]

$$I_s = kcdE_{ex}(\lambda_{ex})E_{em}(\lambda_{ex} + \Delta\lambda) \quad (4)$$

where E_{ex} is the excitation function at the given excitation wavelength ($\lambda_{ex} = \lambda_{em} - \Delta\lambda$), E_{em} is the normal emission function at the corresponding emission wavelength ($\lambda_{em} = \lambda_{ex} + \Delta\lambda$), c is the analyte concentration, d is the thickness of the sample cell, and K is the characteristic constant comprising the "instrumental geometry factor" and related parameters. When $\Delta\lambda = 0$, we can get

$$I_s = kcdE_{ex}(\lambda_{ex})E_{em}(\lambda_{ex}) \quad (5)$$

where I_s is the intensity of synchronous luminescence at $\Delta\lambda = 0$.

As the resonance light scattering is a particular synchronous luminescence, the relationship between the intensity of resonance light scattering (I_{RLS}) and the concentration of the aggregate should follow eq 5. Since the nature of the interaction of the binary complex with nucleic acids is the stacking of the binary complex of the single-stranded nucleic acids, in the case the binary complex is excessive, the concentration of aggregate is equal to the concentration of nucleic acids. The correlation between I_{RLS} and the concentration of nucleic acids can be seen from Table 2. All the regression equations in Table 2 have small intercepts in I_{RLS}, so I_{RLS} is in direct proportion to the concentration of nucleic acids. Those equations, along with limits of detection and precision, were obtained according to the general procedure. The proportional correlation of the enhanced intensity of light scattering with the concentration of calf thymus DNA is in the range 0−400 ng/mL, with those of fish sperm DNA and yeast RNA in the range 0−300 ng/mL. Table 2 presents the parameters involved in the analytical applications of the enhancement effect of nucleic acids on the resonance light scattering of Co(II)/5-Cl-PADAB. Table 3 gives the results of determination for six synthetic samples which were based on the tolerance of foreign coexisting substances presented in Table 1. From Table 3, it can be seen that the values found for the six synthetic samples are identical with the expected ones, and the recovery and relative standard deviation are very satisfactory. Therefore, the resonance light-scattering method of nucleic acids is applicable. Both Tables 2 and 3 show that, by using the resonance light-scattering technique, nucleic acids in nanogram quantities can be determined with high reproducibility, sensitivity, and simplicity.

Based on the fluorescence resonance energy transfer (FRET), fluorometric methods are sensitive for the determination of DNA and RNA,[1−6] but the organic dyes are carcinogenic and difficult for a common lab to obtain. Still more, the limit of detection, for example, that of EB[1e] being 10 ng/mL, is higher than that of our method. Of course, laser-induced fluorescence methods are highly sensitive, but we are not sure; if laser facilities are used, the resonance light scattering technique should be much more sensitive.

CONCLUSION

The enhancement of Rayleigh scattering resulting from the absorption of aggregates is a very common phenomenon and, therefore, may provide important applications in a wide range of areas. Besides the sensitive determination of nucleic acids, we have proved that, with the light-scattering technique, proteins in nanogram quantities can be determined.[19] In addition, the technique can be used to study the mechanism of interactions of porphyrins with nucleic acids or proteins to monitor the formation of the suprahelical helix of nucleic acids.[23] Although the resonance light-scattering technique is in its infancy, it has many potential applications. For example, the experimental approach

(22) Rubio, S.; Gomez-Hens, A.; Vaalcarce, M. *Talanta* **1986**, *33*, 633−640.

(23) Huang, C. Z.; Li, K. A.; Tong, S. Y. *Bull. Chem. Soc. Jpn.*, submitted.

Table 2. Analytical Parameters of the Determination

nucleic acids	linear regression equation (c, ng/ml)[a]	limit of determination (3σ, ng/mL)	r^b	precision at 200.0 ng/mL (%)
calf thymus DNA	$I = 0.1 + 0.238C$	1.4	0.9994	1.9
fish sperm DNA	$I = -1.0 + 0.293\ C$	0.8	0.9996	2.0
yeast RNA	$I = -0.1 + 0.277\ C$	1.3	0.9996	0.8

[a] I is the enhanced intensity of resonance light scattering. [b] r is the correlation coefficient.

Table 3. Results and Determination for Synthetic Samples

nucleic acids contained in samples (amount μg/mL)	main interferences	amount found (μg/mL, $n = 5$)	recovery (%, $n = 5$)[a]	RSD (%)[b]
calf thymus, DNA 3.00	BSA, Ca(II), Mg(II)	2.97	95.6−100.4	0.9
calf thymus DNA 2.00	A, G, C, T, $H_2PO_4^-$	2.06	97.6−106.4	1.2
fish sperm DNA 2.50	HSA, Zn(II), Cd(II), Cu(II)	2.58	94.6−102.6	2.7
fish sperm DNA 2.00	A, G, C, T, $H_2PO_4^-$	2.14	92.0−100.8	2.9
yeast RNA 3.00	γ-IgG, Mn(II), Ni(II), Pb(II)	3.15	90.0−107.6	3.44
yeast RNA 2.50	A, G, C, T, $H_2PO_4^-$	2.45	90.8−101.2	2.9

[a] The recoveries were obtained by adding 0.40 mL of sample solution and 0.40 mL of 2.5 μg/mL standard solution of nucleic acids. [b] RSD is relative standard deviation for five measurements of samples.

can be expanded to time-resolved measurements in the way of time-resolved fluorescence and to the study on the scattering light probe of inhomogeneous systems, particularly biological macromolecular interacting systems. Sensitive determination of antigens in immunochemistry is very crucial. Considering its high sensitivity, the resonance light-scattering technique may have applications in immunochemistry if unlabeled antigen can form a large species with antibody but labeled antigen cannot or if labeled antigen can form a large species with antibody but unlabeled antigen cannot. The formation of the suprahelical helix of nucleic acids in the presence of porphyrins,[23] which leads to strong resonance light-scattering signals being observed, suggests to us that the resonance light-scattering technique may have applications in genetic diagnosis and PCR for the in vitro exponential amplification of specific nucleic acid sequence. We believe that the resonance light-scattering technique, if equipped with laser facilities, will be highly applicable to analytical chemistry and analytical biochemistry.

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