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INSTRUMENTATION, METHODOLOGY,
AND TECHNICAL DEVELOPMENTS

Interaction of Spectral Probe with Biomacromolecule: Safranin T-Nucleic Acid Assembly

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ABSTRACT

The microsurface–spectral correction (MSASC) technique has been described and applied to study the assembly of safranin T on nucleic acids at pH 2.21. The formation of the electrostatic film in nucleic acids causes the aggregation of small molecules and the aggregation obeys the Langmuir isothermal adsorption. The characterization of the assembly complexes was made. Results have showed that the maximal assembly number of ST is 1.62 on each phosphate in deoxyribonucleic acid and 0.84 on each phosphate in ribonucleic acid and the binding constants of the complexes are $K_{\text{DNA-P-ST}} = 2.11 \times 10^4$ and $K_{\text{RNA-P-ST}} = 1.41 \times 10^4\,\text{L mol}^{-1}$. This aggregation was applied to the quantitative detection of nucleic acids in samples with satisfactory results.

Key Words: MSASC technique; Electrostatic film; Langmuir aggregation Safranin T; Nucleic acid.

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INTRODUCTION

The interaction of nucleic acids with small ions and molecules has become increasingly important to the chemist and the physicist. Nucleic acid chemistry is attracting more and more biochemists for research. Understanding the interaction of a spectral probe with nucleic acids can help us to realize the function of such genetic macromolecules and analyze the transmission of the genetic information. The quantitative determination of nucleic acids is also significant in clinical tests and laboratory practice. Continuously, study of nucleic acid is always very hot and active.[1–7] However, the interaction has not been elucidated satisfactorily and earlier observations have not been explained clearly and reasonably, e.g., the Pesavento equation,[8] and Scatchard model.[9]

Some dyes can react with nucleic acids like the adsorption indicators used in the determination of halogen anions. The interaction of nucleic acid with a dye has the same mechanism as the precipitation adsorption reaction. Commonly, nucleic acid molecule owns complex spatial structure. The helix, winding and folds lead of many holes, gullies and grooves. In acidic solutions (pH 2), from the protonation of the bases, the acid nucleic molecule can be viewed as formed positively ($-NH_3^+$) and negatively ($-PO_4^-$) charged films, defining two thin layers of virtual microsurfaces.[10] They will adsorb small cation and anion e.g., Na^+, Cl^-, dye. Because the binding depends on mainly the electrostatic force, it is easy to be destroyed by some factors, e.g., increasing ion concentration of solution and rising temperature. Because of the three dimensional effect, the assembly of small molecules on nucleic acid (M) is in only a monolayer. Thus, the aggregation of a dye (L) in nucleic acids obeys the Langmuir isothermal adsorption.[11] The equilibrium of the L-M mixture is followed:

$$L\,(\text{aqueous phase, } C_L) \Longleftrightarrow ML_N(M \text{ phase, } C_M) \tag{1}$$

The adsorption equation is useful to characterize the L-M complex:

$$\frac{1}{\gamma} = \frac{1}{N} + \frac{1}{NKC_L} \tag{2}$$

where the symbol K is the binding constant and C_L the molar concentration of excess L. N indicates the maximal binding number of L on M and γ is the molar ratio of L adsorbed to M. K is calculated from Eq. (2). Both C_L and γ are calculated by the relations[12]:

$$\gamma = \eta \times \frac{C_{L0}}{C_M} \tag{3}$$

$$C_L = (1 - \eta)C_{L0} \tag{4}$$

where

$$\eta = \frac{A_c - \Delta A}{A_0} \tag{5}$$

where C_M and C_{L0} are the initial molar concentrations of M and L and η indicates the effective fraction of L. The symbols A_c, A_0, and ΔA are the real absorbance

of the *M-L* complex, the measured absorbance of the reagent blank against water and that of the *M-L* solution against the reagent blank at the peak wavelength λ_2. With an increase of L, γ approaches a maximal binding number, N. A_c is calculated by means of[13]:

$$A_c = \frac{\Delta A - \beta \Delta A'}{1 - \alpha\beta} \tag{6}$$

where

$$\alpha = \frac{\varepsilon_{ML_N}^{\lambda 1}}{\varepsilon_{ML_N}^{\lambda 2}} \tag{7}$$

and

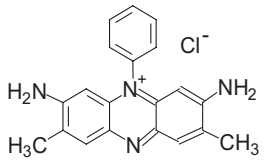
$$\beta = \frac{\varepsilon_{L}^{\lambda 2}}{\varepsilon_{L}^{\lambda 1}} \tag{8}$$

where $\Delta A'$ indicates the absorbance of the *M-L* solution measured at the valley absorption wavelength λ_1. Both α and β are often the correction constants.[12,13] The symbols $\varepsilon_{ML_N}^{\lambda 1}$, $\varepsilon_{ML_N}^{\lambda 2}$, $\varepsilon_{L}^{\lambda 1}$, and $\varepsilon_{L}^{\lambda 2}$ are the molar absorptivities of ML_N and L at wavelengths λ_1 and λ_2 respectively. If the dimer or polymer reaction[15] occurs among L molecules, β changes with C_{L0}. The absorptivity (real $\varepsilon_r^{\lambda 2}$ not apparent $\varepsilon_a^{\lambda 2}$) of the complex at λ_2 is also directly calculated by the relation:

$$\varepsilon_r^{\lambda_2} = \frac{NA_c}{\delta\gamma C_M} \tag{9}$$

where the symbol δ is the cell thickness (cm) and the others have the same meanings as in the equations above. The spectral correction technique is one of the improvement methods for spectrometry but it is different from ordinary method. It can eliminate the absorbance effect of excess of L in the *M-L* solution to give the real absorbance (A_c) of the complex. Therefore, it improves obviously the analytical precision and accuracy of a component traces but also it is very useful to characterize dye-metal,[13] dye-polymer,[14] dye-macromolecule and other reactions. Its combination with Langmuir isothermal adsorption, called microsurface-spectral correction technique (MSASC) will provide a very helpful experimental strategy for study of assembly of a dye on biomacromolecule. In the presented work, safranin T (ST) was selected as a spectral probe to investigate its assembly on nucleic acids (ctDNA and yRNA). Its structure is given below (see Sch. 1).

This dye was earlier applied to the determination of Bi[16], Pd[17], and REE[18]. In acidic solution, it forms trivalent cation because of the protonation of two $-NH_2$ groups. The trivalent ST can be adsorbed strongly on the negatively electrostatic film of the double helix of nucleic acid. The assembly of ST on nucleic acids obeys the Langmuir isothermal adsorption. Results have shown that the binding ratio is followed: ctDNA-P:ST = 1.62 and yRNA-P:ST = 1:0.84. The binding constants of the complexes are $K_{\text{DNA-P-ST}} = 2.11 \times 10^4$ and $K_{\text{RNA-P-ST}} = 1.41 \times 10^4\,\text{Lmol}^{-1}$ and their absorptivities $\varepsilon_{\text{DNA-P-ST}} = 1.49 \times 10^4$ and $\varepsilon_{\text{RNA-P-ST}} = 0.54 \times 10^4\,\text{L mol}^{-1}\,\text{cm}^{-1}$ at 565 nm, respectively. The quantitative determination of nucleic acid in samples has been carried out with the satisfactory result.



Safranin T, water soluble

Scheme 1. Safranin T, water soluble

EXPERIMENTAL

Materials

Absorption spectra were recorded with a UV/VIS 265 spectrophotometer (Shimadzu, Kyoto, Japan). The pH of the solution was measured with a pHS-2C acidity meter (Leici Instruments, Shanghai, China). The temperature was adjusted and remained constant in a Model 116R electric heated thermostatic bath (Changjiang Test Instruments of Tongjiang, China).

Calf thymus deoxyribonucleic acid (ctDNA) purchased from Sigma Chemicals and yeast ribonucleic acid (yRNA) from Shanghai Chemical Reagents of Chinese Medicine Group. Using a standard procedure previously described[2] and stored in 1 mM phosphate buffer, pH 7, containing 10 mM NaCl. The molar concentration of DNA 0.500 mg/L in weight concentration was obtained via absorbance measurement using $\varepsilon_{DNA} = 6600\,M^{-1}\,cm^{-1}$ at the maximum near 260 nm (i.e., nucleic acid concentration is reported in molar base pairs).[19] Results show that their P molarities are 1.13 and 1.36 mM, respectively. ST solution (1.00 mM) was prepared by dissolving 0.3693 g of safranin T (content 95%, Shanghai Chemical Reagents) in 1000 mL of deionized water. Britton–Robinson buffer solutions (between pH 2.21 and 11.6) were used to adjust the acidity of the reaction solution. 2 M NaCl was added to adjust ionic strength of the aqueous solution. Na_2-EDTA solution (1%) was prepared to mask foreign metal ions existed possibly in samples. All reagents were of analytical grade and used without further purification.

Methods

Interaction of ST with Nucleic Acids.

Into a 10 mL calibrated flask were added an appropriate working solution of nucleic acids, 1 mL of Britton–Robinson buffer solution and appropriate ST solution. The mixture was then diluted with deionized water to 10 mL and mixed thoroughly. After 5 min, the absorbances were measured at 565 and 520 nm against the blank treated in the same way without any nucleic acids and then A_c, η, γ, and C_L were calculated.

Quantitative Determination of Nucleic Acid in Samples.

The masking reagent, 0.5 mL of Na_2-EDTA solution (1%) was added to complex metal ions possibly existed in samples e.g., Fe(II, III), Ca(II), Zn(II), Mg(II), Pb(II). The next successive operation is same as the above procedure. Finally A_c was calculated.

RESULTS AND DISCUSSION

Spectral Analysis

The interaction of ST with nucleic acid (ctDNA as representative) was carried out. At pH 2.21, the absorption spectra of the ST-ctDNA solutions are shown in Fig. 1. From curves 1, the absorption peak of ST is located at 515 nm. From curve 2, the absorbance ratio of the ctDNA-ST solutions approaches a minimum and remains constant when the ratio of ctDNA to ST is over 1.12 mg/μmol (ctDNA-P:ST = 2.7:1 μmol/μmol). So the solution containing over 1.2 mg of ctDNA and 0.50 μmol of ST is composed of only DNA-ST complex and excess DNA but not any longer of free ST. Curve 3 gives the spectrum of such a solution. The peak of the ctDNA-ST complex is located at 525 nm. By comparing curve 1 with 3, the spectral red shift of the complex is only 10 nm. From the relative spectra 4–7 of the various pH solutions, we find that curve 4 gives the maximal peak

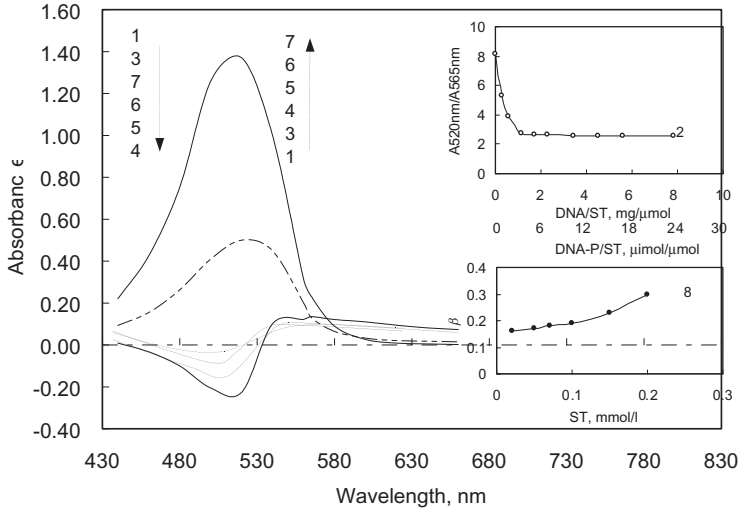


Figure 1. Absorption spectra of the ST-ctDNA solutions (10 mL) and others: 1-ST (0.500 μmol) solution at pH 2.21, 2—variation for the two absorbance ratio of solutions at 520 and 565 nm with ratio of ctDNA to TN at pH 2.21, 3—ST (0.500 μmol)- ctDNA (1.12 mg) at pH 2.21, 4—ST (0.500 μmol)-ctDNA (0.250 mg) at pH 2.21, 5—, 6—, 7— all same as 4—but at pH 3.29, 6.37, and 7.96, respectively, 8—variation of β with ST concentration at pH 2.21, all 1, 2, 3, and 8 against water and the others against the reagent blank.

at 565 nm and valley at 520 nm. Those two wavelengths were used to characterize the nucleic acids complex with ST in pH 2.21 solution. From both curves 2 and 3, the correction coefficient was calculated by Eq. (7) to be $\alpha = 2.63$. From curve 8, β changes with ST concentration, which is calculated by Eq. (8). ST self-aggregation occurs to form a dimmer or polymer because β increases with increase of ST.

Effect of Operation Conditions (pH, Ionic Strength, and Temperature)

By varying pH of solution, the absorbance of the ctDNA-ST solution was measured and the binding ratio of ST to ctDNA is shown in Fig. 2. From curve 1, we observe that the interaction of ST with ctDNA is more sensitive in acidic solution. This is attributed to the formation of ST^{3+} by protonation of $-NH_2$ in acidic solution and it is attracted easily into nucleic acid. In this work, pH 2.21 buffer solution was used.

The influence of ionic strength on the binding ratio is shown in Fig. 2. From curve 2, γ decreases with increase of ionic strength between 0 and 0.4 M. This is attributed to the fact that much more Na^+ than ST is attracted to screening the activated microsurface of DNA.

From curve 3, γ decreases slowly with increase in temperature between 20 and 60°C. The binding ratio decreases by only 0.075 per increasing 10°C. This is attributed to the two synergistic actions: high temperature causes a rapid desorption of ST from the activated microsurface of DNA and trivalent ST^{3+} can bind on nucleic acids by a strong attraction. At room temperature, the interaction of ST with nucleic acid is complete in 2 min.

Effect of ST and Characterization of Complexes

By varying ST concentration, the ST-DNA and ST-RNA assembly were measured. γ and C_L were calculated in each case and their relationship is shown in Fig. 3. Both of them are linear, so the aggregation of ST on nucleic acids obeys the Langmuir isothermal adsorption. From the intercepts of lines, the maximal assembly number of ST on ctDNA-P and yRNA-P are calculated to be 1.62 and 0.84. From the slopes, the binding constants of the complexes were calculated to be $K_{\text{DNA-P-ST}}$ 2.11×10^4 and $K_{\text{RNA-P-ST}}$ 1.41×10^4 L mol^{-1}. From Eq. (9), their molar absorptivities were calculated to be $\varepsilon_{\text{DNA-P-ST}} = 1.49 \times 10^4$ and $\varepsilon_{\text{RNA-P-ST}} = 0.54 \times 10^4$ L mol^{-1} cm^{-1} at 565 nm. The maximal assembly number of ST on ctDNA is almost double that on yRNA because the content in double helix of RNA only about 50–60% that of DNA. In characterization of a biomacromolecular complex, the spectral correction method has special advantages in operation and principle by contrast of classical methods because it eliminates the effect of excess of dye in the mixed solution. Most of the classical spectrometric methods, e.g., the molar ratios, equilibrium movements, Pesavento Equation (8) are often applied to characterization of a metal complex with ligand which has no absorption at work wavelength.

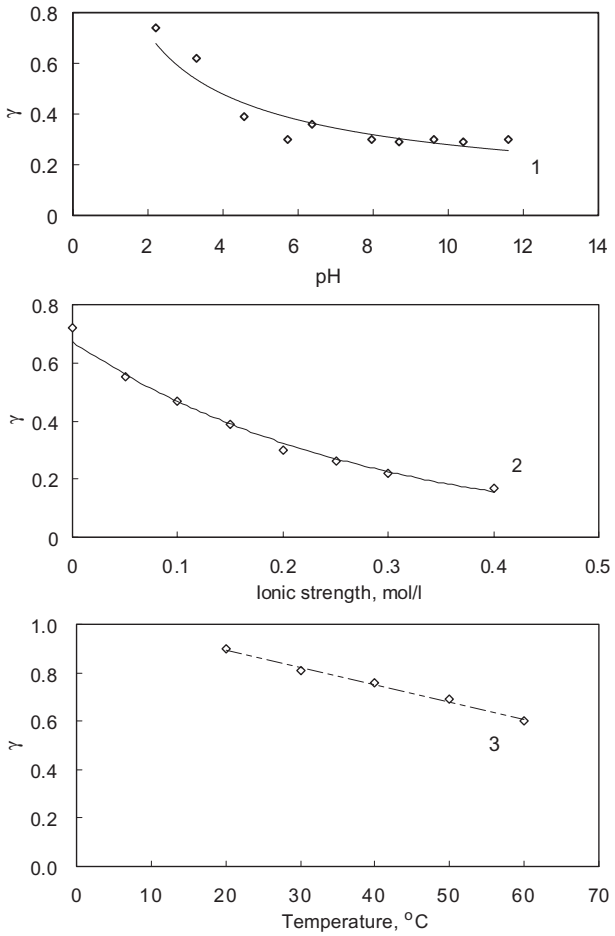


Figure 2. Effect of pH (1), ionic strength (2), and temperature (3) on γ, where the solutions (10 mL) contain 0.500 μmol of ST and 0.250 mg of ctDNA.

Quantitative Detection of Nucleic Acids

Calibration Graph and Precision

The standard series of nucleic acids (0–0.40 mg/10 mL) were prepared and measured at pH 2.21, where 0.50 mL of 1.00 mmol/L ST was added. The regression equations are given in Table 1. We find that plots A_c vs. x lines are always more linear than plots ΔA vs. x and the former slopes are always higher than the latter ones. Therefore, the spectral correction method is more precise and more accurate than ordinary spectrophotometry because it eliminates the effect of free ST in the mixed solution. For 0.010 of A_c, the detection limit of nucleic acid was calculated to be 8 μg of ctDNA and 15 μg of yRNA in 10 mL of solution. Eight replicated determinations of 0.150 mg of ctDNA and 0.150 mg of yRNA were made by the

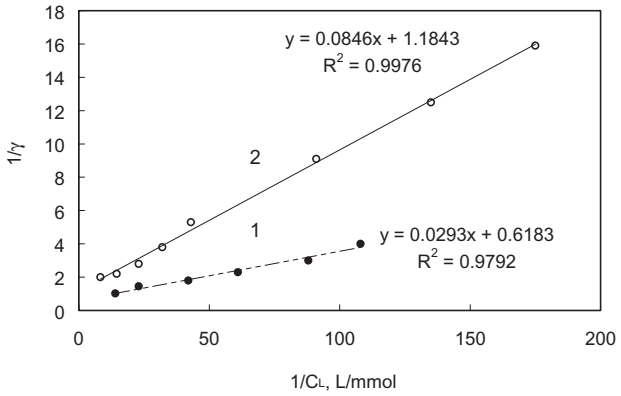


Figure 3. Plots γ^{-1} vs. $C_L{}^{-1}$: 1—ctDNA (0.280 mg)-ST solution (10 mL), 2—yRNA (0.250 mg)-ST solution (10 mL).

Table 1. The regression equations for the determination of nucleic acids with ST at pH 2.21.

Determination	Linear scope mg/10 mL	Regression equation at 565 nm	Correlation coefficient
ctDNA	0–0.40	$A_c = 1.23 \times -0.003$	0.9982
		$\Delta A = 0.514 \times -0.003$	0.9896
yRNA	0–0.40	$A_c = 0.601 \times -0.0002$	0.9981
		$\Delta A = 0.206 \times +0.005$	0.9572

spectral correction method. Their means are $0.154 + 0.007$ mg and $0.143 + 0.006$ mg, respectively and the relative standard deviations (RSD) 4.5% and 4.6%. However, their means are $0.176 + 0.027$ mg and $0.168 + 0.036$ mg by the single-wavelength spectrophotometry and the RSDs 15% and 21%. Therefore, the spectral correction method is more accurate and moe precise than ordinary spectrophotometry.

Effect of Foreign Ions

By adding EDTA-Na_2 in the ST-ctDNA solution, we observed that none of the following substances and ions affected the direct determination of 0.250 mg of ctDNA (less than 10% error): 1 mg of K^+, NH_4^+, Ca(II), SO_4^{2-}, 0.5 mg of Cl^-, F^-, PO_4^{3-}, $C_2O_4^{2-}$, I^-, Ac^-, glucose, amino acid, Mg(II), Al(III), Fe(III), 0.1 mg of acetone, ethanol, Cu(II), Mn(II), Zn(II), Pb(II), Ni(II), Co(II), Cd(II) and 0.02 mg of Hg(II).

Preparation and Analysis of Samples

The synthesis of two samples was made for examining the recovery of nucleic acids. They were prepared in drinking water and lake water background. Into each sample, added 1 mg of Mg(II), acetone, glucose, amino acid, sugar, F^-, protein;

Table 2. Determination of nucleic acids in samples (10 mL) in the presence of Na_2EDTA.

Sample no.	Added (mg)	Found (mg) and recovery
1#: drinking water background (three replicated determinations)	ctDNA 0.100 yRNA 0.100	0.106 ± 0.007 rec. 106% 0.093 ± 0.005 rec. 93%
2#: lake water background (three replicated determinations)	ctDNA 0.100 yRNA 0.100	0.099 ± 0.008 rec. 99.0% 0.098 ± 0.007 rec. 98.0%

0.3 mg of Mn(II), Zn(II), Fe(III) and 0.03 mg of Pb(II), Cd(II) and Hg(II). Drops of the standard ctDNA or yRNA solution were added. The determination results are given in Table 2. We observe that the recovery of nucleic acids was between 93.0 and 106% and RSD less than 8.1%.

CONCLUSIONS

Spectrophotometry is classical but still very useful for determination of a component traces, especially in morphological analysis of a compound[21–25] and investigation of a chemical interaction mechanism.[10,20] The investigation to the interaction of ST with nucleic acids supports the Langmuir monolayer adsorption of dye on the virtual charged film surface of biomacromolecule. Though the MSASC technique is not sensitive enough, it does improve both the precision and accuracy of trace analysis and offer the additional benefits of simplicity and versatility.

ABBREVIATIONS

ctDNA	Calf thymus deoxyribonucleic acid
DNA	Deoxyribonucleic acid
MSASC	Microsurface-spectral correction technique
RSD	Relative standard deviations
ST	Safranin T
UV/VIS	Ultraviolet/visible
yRNA	Yeast ribonucleic acid

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