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Investigation of Interaction of Proflavine and Naphthol Red in Nucleic Acids by Light-Absorption Spectrometry

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ABSTRACT

The formation of the double electrostatic films in nucleic acid will cause the aggregation of small molecules on it by a non-covalent bond. The interaction of the dyes: proflavine (PLV) and naphthol red (NR) with nucleic acids [calf thymus (ct) DNA and yeast (y)RNA] in acidic medium was investigated by the microsurface adsorption–spectral correction (MSASC) technique. Results showed that the aggregation of PLV and NR on DNA and RNA agrees with the Langmuir isothermal adsorption. The maximal binding numbers of PLV is 0.85 with ctDNA-P and 0.50 with yRNA-P and those of NR 0.12 with ctDNA-P and 0.07 with yRNA-P. Their binding constants K are 8.13×10^4, 2.15×10^5, 2.20×10^5, and 2.05×10^5, respectively. The effect of ionic strength and temperature on the aggregation was made. The aggregation of PLV and NR in nucleic acids has been tried to apply to the quantitative detection of nucleic acids.

Key Words: MSASC technique; Electrostatic film; Proflavine; Naphthol red; Nucleic acid, Light-absorption spectrometry.

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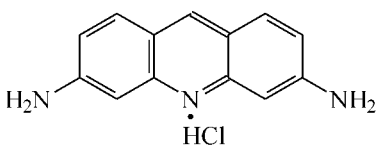
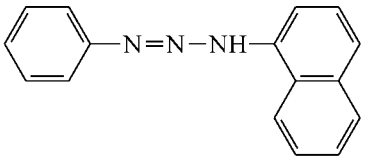
INTRODUCTION

Chemists are always interested in biomacromolecular assembly.[1–5] In order to accurately analyze the physico-chemical action and process occurring inside biological cell, the interaction of small molecules with biomacromolecule is often investigated in the laboratory. In fact, the molecular interaction is very complicated, which includes chemical bond connection e.g., covalent bond, coordinate bond and physical force connection e.g., electrostatic attraction, hydrogen bond, van der Waals force, hydrophobic bond, insertion, winding. Understanding the chemical, physical, biological, and pharmacological activity of a complex system requires knowledge of the state of molecular aggregation of the system's components. The electrostatic attraction as a source force shortens the distance between different molecules with charging or polarizable atoms then to induce the non-covalent connection among molecules. The physical action is often weaker than a chemical bond so it is broken easily by high temperature or by high ionic strength. Molecular spectrometry is extensively used in the study of biomacromolecular assembly[1–5] and in determination of biopolymer, e.g., DNA.[6,7] Study of nucleic acids is always very active.[8–13] The quantitative analysis of nucleic acids is important in clinical tests and laboratory practice. The interaction of small molecules with nucleic acids has not been elucidated satisfactorily and earlier observations have not been explained clearly though a number of models were early proposed, e.g., the Pesavento equation,[14] and Scatchard model.[15] The coupling of both the microsurface adsorption and the spectral correction technique will provide a very helpful experimental strategy for study of chromophore or its metallic complex's adsorption in surfactant solution. This method is named Micro Phase Adsorption-Spectral Correction named as MSASC[16] and provides a useful experimental strategy for the study of aggregation of small molecules on biopolymer. In the present work, the assembly of proflavine (PLV) and naphthol red (NR) in nucleic acids was investigated by MSASC. The structure of the dyes are given below:



(1) Proflavine (PLV)



(2) Naphthol red (NR)

Proflavine forms the bivalent cation and NR the univalent cation in acidic solution due to the protonation of $-NH_2$ and $-NH$-groups so they may bind on nucleic acids. They were ever used in recognition of DNA sequences and molecular spectrometric analysis.[17–24] This work is to mainly study their connection with DNA and RNA from another aspect by light-absorption spectrometry. The operation is simple and the principle is understandable. The aggregation of PLV and NR in nucleic acids obeys the Langmuir isothermal adsorption. Results show that the binding numbers of PLV and NR to calf thymus (ct) DNA-P is 0.85 and 0.12 and those of PLV and NR to yeast (y) RNA-P 0.5 and 0.07. Their binding constants are $K_{\text{ctDNA-PLV}} = 8.13 \times 10^4$, $K_{\text{yRNA-PLV}} = 2.15 \times 10^5$, $K_{\text{ctDNA-NR}} = 2.20 \times 10^5$ and $K_{\text{yRNA-NR}} = 2.05 \times 10^5\,\text{L mol}^{-1}$, respectively and their molar

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adsorptivities 1.04×10^4, $4.59 \times 10^3\,L\,mol^{-1}\,cm^{-1}$ at 480 nm and 3.35×10^3, $1.36 \times 10^3\,L\,mol^{-1}\,cm^{-1}$ at 600 nm. Because of the non-covalent bond binding, the binding number of PLV and NR on DNA decreases with an increase of ionic strength and temperature.

EXPERIMENTAL

Apparatus and Materials

Absorption spectra were recorded on TU1901 Spectrophotometer (PGeneral, Beijing). The conductivity meter, Model DDS-11A (Tianjin 2nd Anal. Instruments, Tianjin, P.R. China) was used to measure conductivity together with Model DJS-1 conductivity immersion electrode (electrode constant 0.98, Shanghai Tienkuang Devices, Shanghai, P.R. China) in production of deionized water below $0.5\,\mu\Omega\,cm^{-1}$. pH of solution was measured on a pHS-2C acidity meter (Leici Instruments, Shanghai) and Model 620D pH Pen (Shanghai Ren's Electronic). The temperature was adjusted and remained constant in a electronic heated thermostat bath, Model 116R (Changjiang Test Instruments of Tongjiang, Hebei State, P.R. China).

Preparation of Solutions

Calf thymus (ct) DNA was purchased from Sigma Chemicals and yeast (y) RNA from Shanghai Chemical Reagents of Chinese Medicine Group. Using a standard procedure previously described[9] and stored in 1 mM phosphate buffer, pH 7, containing 10 mM NaCl. The molar concentrations of ctDNA and yRNA (both $0.500\,mg\,L)^{-1}$ in weight concentration were obtained via absorbance measurement using $\varepsilon_{ctDNA} = 6600\,M^{-1}\,cm^{-1}$ and $\varepsilon_{yRNA} = 7800\,M^{-1}\,cm^{-1}$ at the maximum near 260 nm (i.e., nucleic acid concentrations are reported in molar base pairs).[25] The organic dye solutions, PLV (PLV, 3,6-diaminoacridine, were made in Schmid GMBH, Germany) and NR (NR, was made in B.D.H. Laboratory Chemicals, UK) purchased from Shanghai Chemical Reagents and it was dissolved in deionized water. The concentrations of PLV and NR were 1.000 and 0.500 mM, respectively. The Britton–Robinson buffer solutions (pH 2.21–8.69) were used to control the acidity. NaCl (2 M) was used to adjust the ionic strength of the aqueous solutions. Na_2-EDTA solution (1%) was prepared to mask the foreign metallic ions co-existed possibly in the practical samples. All reagents were of analytical grade and used without further purification. The surfactant solutions, Triton x-100, sodium dodecyl benzene sulfonate (SDBS) and cetyltrimethylammonium bromide (CTAB) (all 1%) were prepared for studying the effect on sensitivity and as interference substances.

Measurements

Interaction of Dyes with Nucleic Acids

Into a 10 mL calibrated flask were added an appropriate working solution of nucleic acids or nucleic acids, 1.0 mL of Britton–Robinson buffer solution and appropriate PLV

or NR solution. The mixture was then diluted with deionized water to 10 mL and mixed thoroughly. All the absorption measurements were obtained against the blank treated in the same way without nucleic acids.

Preparation of Samples and Quantitative Determination of Nucleic Acid

The synthesis of two samples was made for the recovery of nucleic acids. In the first sample (1#), 1 mg of Ca(II), acetone, glucose and PO_4^{3-}, BSA, 0.1 mg of F^-, Cu(II), Mn(II), Zn(II), 0.05 mg of Pb(II) and Cd(II) were added. In the other sample (2#), 2 mg of NH_4^+, ethanol, NO_3^-, amino acid, sugar, 0.2 mg of F^-, Fe(II), Mg(II), Zn(II), 0.05 mg of Pb(II), Ni(II) and 0.01 mg of Hg(II) were added. Drops of the standard ctDNA and yRNA solutions were added in the first sample and the other sample, respectively. The first was in a lake water background and the other is in a drinking water background. In the determination of nucleic acids, 0.5 mL of Na_2-EDTA solution (1%) was added to complex metal ions. The successive next operation was made according to the same procedures as above.

Principal Equations and Calculation

The interaction of proteins with dyes was investigated early.[26] Nucleic acid molecule also possesses a complex spatial structure. The protonation of $-NH_2$ and $-NH$ of the bases forms the positive electrostatic charges film at the side of the double helix and the dehydrophosphate presents a negative electrostatic charges film at the other side. Thus, the double electrostatic films can attract small charged molecules, e.g., dye ions and then coaction of the other non-covalent bonds, e.g., hydrogen bond, van der Waals force, hydrophobic bond, insertion, and molecular winding results in the firm binding of small organic molecule in DNS. Therefore, the electrostatic attraction is primary and original force of the non-covalent connection. The non-covalent binding is in accords with the Langmuir isothermal adsorption so it may be regarded as an adsorption reaction in only a monolayer. The Langmuir equation is used as followed:[27]

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

where K is the binding constant and C_L-the molar concentration of the free L. N indicates the maximal binding number of L in M and γ is the molar ratio of the effective L to M. Both C_L and γ are calculated by means of:[26,28]

$$\gamma = \eta \times \frac{C_{L0}}{C_M} \tag{2}$$

$$C_L = (1 - \eta)C_{L0} \tag{3}$$

where

$$\eta = \frac{A_c - \Delta A}{A_0} \tag{4}$$

where both C_M and C_{L0} are the molar concentration of M and L initially and η indicates the effective fraction of L. A_c, is the real absorbance of the M-L aggregate and A_0 and

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ΔA are the absorbances of L and that of the M-L solution, respectively measured at peak wavelength λ_2 against water and a reagent blank. Within the increase in L molar concentration, γ will approach a maximum N. A_c is calculated by the relation:[29]

$$A_c = \frac{\Delta A - \beta \Delta A'}{1 - \alpha\beta} \tag{5}$$

where $\Delta A'$ indicates the absorbance of the M-L solution measured at the valley absorption wavelength λ_1. Usually, α and β are the correction constants[27] and they are calculated by measuring directly ML_N and L solutions. In addition, the molar absorptivity (real $\varepsilon_r^{\lambda_2}$ not apparent $\varepsilon_a^{\lambda_2}$) of the adsorption product ML_N at λ_2 is also directly calculated by the means of:

$$\varepsilon_r^{\lambda_2} = \frac{NA_c}{\delta\gamma C_M} \tag{6}$$

where δ is the cell thickness (cm) and the others have the same meanings as in the equations above.

RESULTS AND DISCUSSION

Spectral Analysis

The interactions of PLV and NR with nucleic acid (ctDNA as representative) were made and their absorption spectra are shown in Fig. 1. The aggregates have longer absorption peak wavelength than the dyes. From curves 1, 2, 4, and 5, the peak absorption of the PLV, PLV-DNA aggregate, NR and NR-DNA aggregate are located at 450, 465, 530, and 540 nm, respectively. The spectral red shift of the PLV-DNA aggregate was only 15 nm and that of the NR-DNA aggregate only 10 nm. However, from relative spectra 3 and 6, the peak of the ctDNA-PLV solution was located at 480 nm and the valley at 430 nm and the peak of the ctDNA-NR solution at 600 nm and the valley at 540 nm, respectively. The four wavelengths were used in this work so as to bring the sensitive measurement. The spectral correction method was used instead of ordinary spectrophotometry because of the little spectral shift. From curves 2 and 5, the correction coefficient of the PLV-ctDNA and NR-ctDNA aggregates is calculated to be $\alpha_{PLV\text{-}DNA} = 0.850$ and $\alpha_{NR\text{-}DNA} = 1.64$. Curves a-2 and b-2 indicate β variations of the only PLV and only NR solutions. β decreases slowly with increase in PLV and NR concentration. This is attributed to the self-aggregation of PLV and NR themselves in the solutions.

Effect of pH

By varying pH of solution, the absorption of the ctDNA-PLV and ctDNA-NR solutions were measured and the ratio of PLV and NR to ctDNA is shown in Fig. 2. The lower the pH is and the higher the binding ratio becomes. This is attributed to the fact that the PLV and NR ions were formed easily in acidic solution because of the protonation of $-NH_2$ and $-NH$ and they were adsorbed closely in the grooves of nucleic acids.

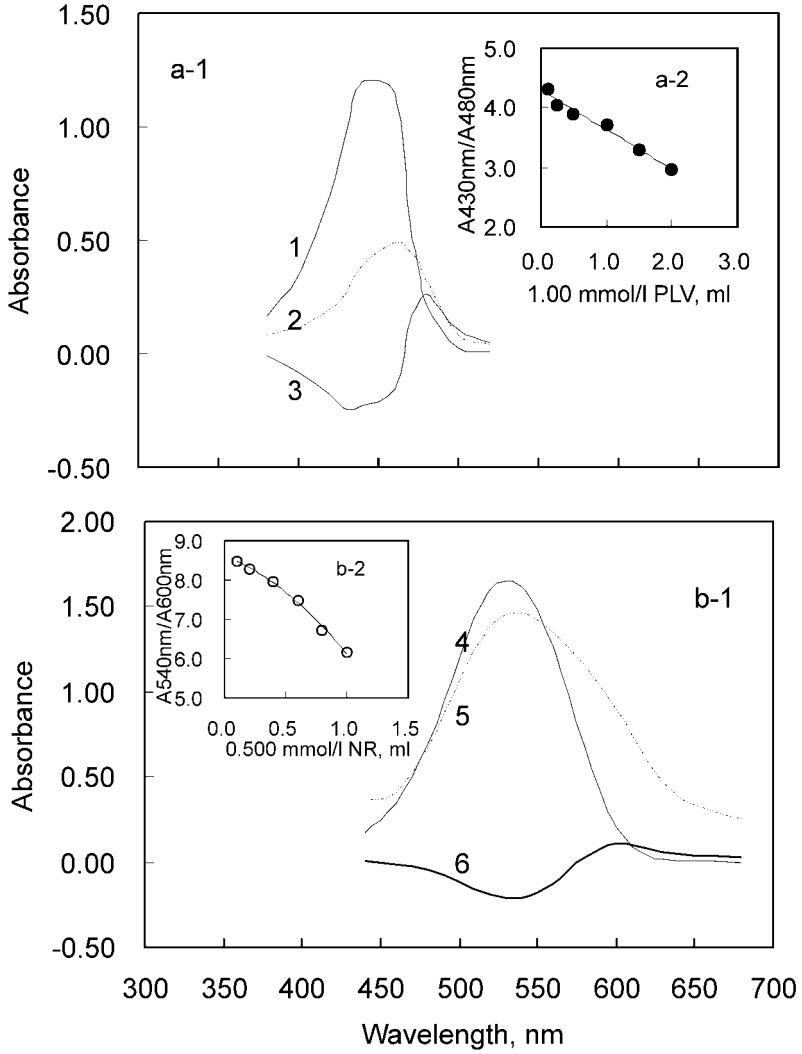

Figure 1. Absorption spectra of PLV (a-1), NR (b-1) and their DNA solutions and variation of β with PLV (a-2) and NR (b-2) concentrations at pH 2.21: 1—0.500 μmol of PLV, 2—0.500 μmol of PLV plus 2 μmol of DNA-P, 3—0.500 μmol of PLV plus 0.250 mg of DNA, 4—0.250 μmol of NR, 5—0.250 μmol of NR plus 2 μmol of DNA-P, 6—0.250 μmol of NR plus 0.250 mg of DNA. Only 3 was against the reagent blank without DNA contained and the others against water.

Characterization of Assembly

By varying the addition of PLV and NR solutions, the absorption of nucleic acid interaction solutions was measured. γ and C_L of each solution were calculated and their relationship is shown in Fig. 3. From Fig. 3a, the binding ratio of PLV to ctDNA and yRNA approaches the maximum at 0.85 and 0.46 when the addition of the PLV solution

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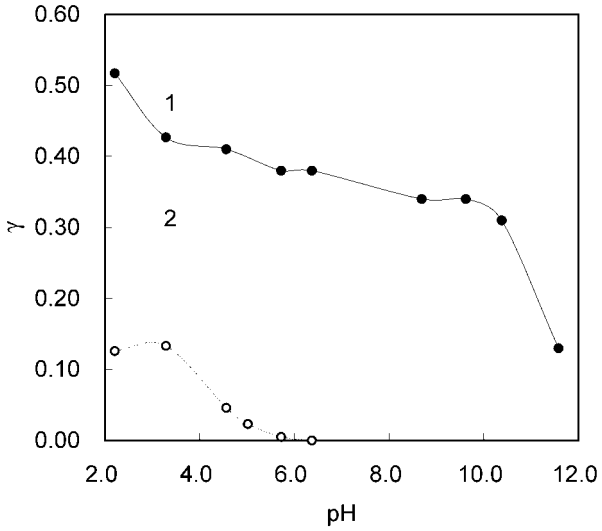

Figure 2. Effect of pH on the binding ratio of PLV to DNA and that of NR to DNA in the interaction solution: 1—0.500 μmol PLV plus 0.250 mg of DNA and 2—0.250 μmol of NR plus 0.250 mg of DNA.

is over 0.70 mL. Similarly, from Fig. 4b, the binding ratio of NR to ctDNA and yRNA approaches the maximum at 0.12 and 0.07 when the addition of NR solution is over 0.50 mL. From Fig. 4, plots γ^{-1} vs. ${C_L}^{-1}$ are all linear. Therefore, the interaction of PLV and RN with DNA and RNA obeys the Langmuir isothermal adsorption. The linear regression equations are given below:

$$\frac{1}{\gamma} = 1.18 + \frac{1.45 \times 10^{-5}}{c_{\text{ctDNA}}} (\text{ctDNA–PLV}) \quad \text{and}$$

$$\frac{1}{\gamma} = 2.01 + \frac{0.93 \times 10^{-5}}{C_{\text{yRNA}}} (\text{yRNA–PLV})$$

$$\frac{1}{\gamma} = 8.32 + \frac{3.79 \times 10^{-5}}{c_{\text{ctDNA}}} (\text{ctDNA–NR}) \quad \text{and}$$

$$\frac{1}{\gamma} = 14.2 + \frac{6.95 \times 10^{-5}}{C_{\text{yRNA}}} (\text{yRNA–NR})$$

From the intercepts of lines, the maximal binding number of PLV on ctDNA and yRNA is 0.85 and 0.50 and the binding constants of the aggregates were calculated to be $K_{\text{DNA-PLV}} = 8.13 \times 10^4$ and $K_{\text{RNA-PLV}} = 2.15 \times 10^5\,\text{L mol}^{-1}$. At the same method, the maximal binding number of NR on ctDNA and yRNA is 0.12 and 0.07 and $K_{\text{DNA-NR}} = 2.20 \times 10^5$ and $K_{\text{RNA-NR}} = 2.05 \times 10^5\,\text{L mol}^{-1}$. The binding numbers of PLV on DNA and RNA are always more than those of NR on DNA and RNA. This is attributed to the fact that PLV molecule is parallel to the helix groove across 2–3 base

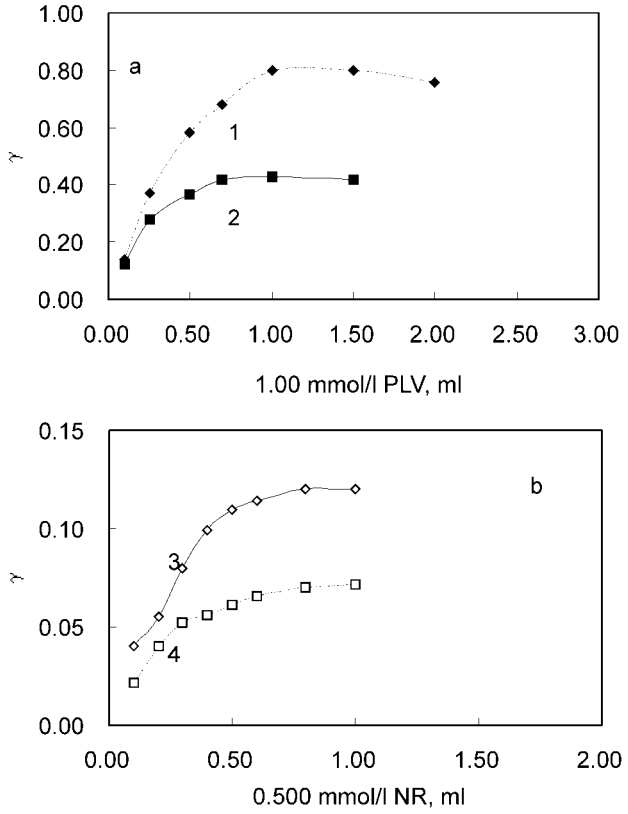

Figure 3. Effect of addition of the PLV (a) and NR (b) solution on the binding ratio of: 1—PLV to DNA, 2—PLV to RNA, 3—NR to DNA, and 4—NR to RNA.

pairs and NR is parallel to the base pair chain across the groove. Thus, NR has a greater space steric effect than PLV. Besides, PLV carries more positive charges than NR. By comparing their *K*s, the DNA-NR and RNA-NR complexes are more stable than the DNA-PLV and RNA-PLV complexes. Besides the electrostatic attraction, the other non-covalent binding forces between NR and the bases, e.g., hydrogen bond, hydrophobic bond, insertion and molecular winding are stronger than that between PLV and the bases. In addition, the parallel array of the PLV molecules along the groove enhances the repulsive interaction among the positively charged PLV molecules. The molar absorptivities of the PLV-DNA and PLV-RNA aggregates were calculated to be $\varepsilon = 1.04 \times 10^4$ and $4.59 \times 10^3\,\mathrm{L\,mol^{-1}\,cm^{-1}}$ at 480 nm and those of the NR-DNA and NR-RNA to be 3.35×10^3 and $1.36 \times 10^3\,\mathrm{L\,mol^{-1}\,cm^{-1}}$ at 600 nm. In the determination of the binding ratio and property constant of a aggregate, the spectral correction technique has special advantage in operation and principle by contrast of classical methods such as Scatchard model,[15] molar ratios, and others. Additionally, because the binding number of PLV and NR in RNA approaches 0.6 times that of DNA, the double-helix chain length of RNA is estimated about 60% that of DNA.

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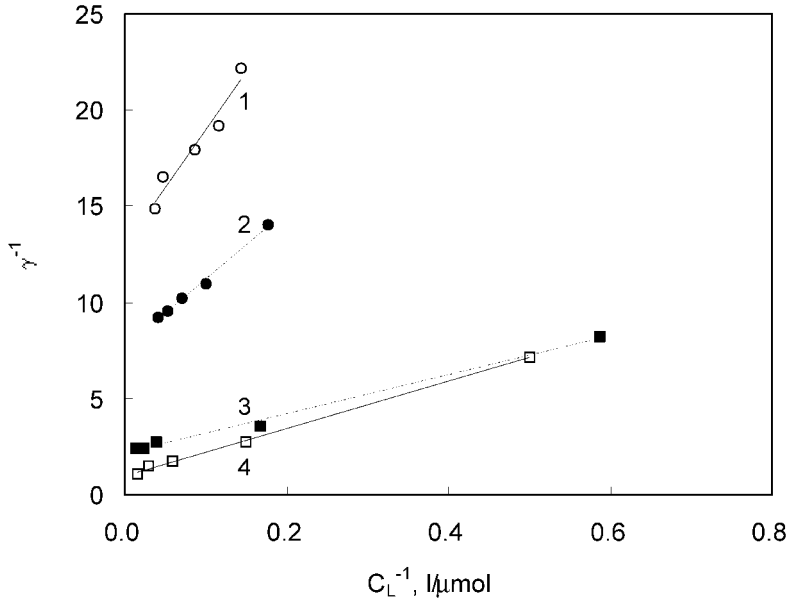

Figure 4. Plot C_L^{-1} vs. γ^{-1}: 1—ctDNA–PLV, 2—yRNA–PLV, 3—ctDNA–NR, and 4—yRNA–NR.

Effect of Temperature, Ionic Strength, and Various Surfactants

The variation of the binding ratio of PVA and NR to ctDNA (as representative) is shown in Fig. 5(a) γ decreases rapidly with increase in temperature from curve 1, but γ decreases slightly from curve 2. This is attributed to the fact that the non-covalent binding forces between NR and the bases are stronger than that between PLV and the bases as described in the last paragraph. At room temperature, the interaction of PLV and NR with nucleic acid is complete in 10 min.

The influence of ionic strength of solution on the binding ratio is shown in Fig. 5(b). Between ionic strength 0 and 0.16 M, the binding ratios of PLV and NR to DNA decrease with increase in ionic strength, especially in the PLV-DNA solution. This is attributed to the fact that much more Na^+ than PLV or NR will bind on DNA to substitute the dyes.

From Fig. 5(c), the addition of ionic surfactants both CTAB and SDBS results in the serious influence on the interaction of the dyes with nucleic acid. This is attributed to the strong adsorption of the dyes on SDBS and the strong interaction of CTAB with DNA to replace the dyes. On the contrary, non-ionic surfactant Triton x-100 hardly affects the interaction of the dyes with nucleic acid.

Application to Determination of Nucleic Acid

Calibration Graph and Precision

The interaction of PLV and NR with nucleic acids was tried to the quantitative analysis of nucleic acid. The standard series of nucleic acids were prepared and measured

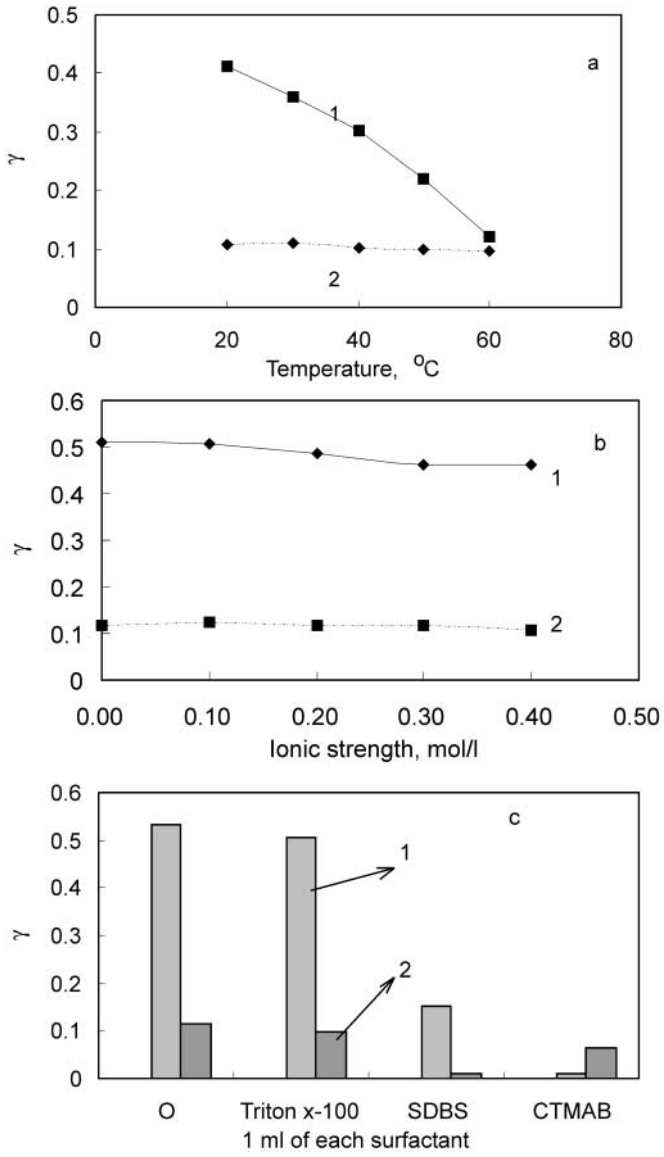

Figure 5. (a) Effect of temperature, (b) ionic strength, and (c) surfactants on the binding ratio of PLV to DNA and that of NR to DNA (0.250 mg): 1—PLV (0.700 μmol) and 2—NR (0.300 μmol P).

at pH 2.21. The regression equations are given in Table 1. By comparing the slopes, the slopes of RNA are about 0.6 times that of DNA. For 0.010 of A_c, the detection limit of nucleic acid was calculated to be 0.010 mg with PLV as reactant and 0.020 mg with NR as reactant both in 10 mL solution. Seven replicated determinations of 0.300 mg of DNA were carried out with PLV as reactant. The mean result is 0.290 $\pm$ 0.010 mg, the recovery 96.7% and the relative standard deviation (RSD) 4.13%. Similarly, when NR was used

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Table 1. The linear regression equations for the determination of nucleic acids with PLV and NR as reactants at pH 2.21 in the presence of EDTA.

Determination	Linear scope (mg 10 mL^{-1})	Regression equation (x: μmol P)	Correlation coefficient
ctDNA (with PLV)	0–0.40	$A_c = 1.36x + 0.021$	0.9951
yRNA (with PLV)	0–0.40	$A_c = 1.05x + 0.014$	0.9971
ctDNA (with NR)	0–0.50	$A_c = 0.482x + 0.002$	0.9990
yRNA (with NR)	0–0.50	$A_c = 0.334x - 0.006$	0.9979

in place of PLV, the mean is 0.116 ± 0.012 mg of DNA, the recovery 116% and the RSD 4.1%.

Effect of Foreign Ions

By addition of EDTA-Na_2 (0.5 mL of 1%) in the solution, the influence of foreign substances and ions was investigated at pH 2.21 and none of the following compounds or ions affected the direct determination of 1.00 μmol of DNA (less than 10% error): 2 mg of K^+, NH_4^+, Cl^-, 1 mg of F^-, PO_4^{3-}, $C_2O_4^{2-}$, glucose, amino acid, Mg(II), Ca(II), Fe(III), 0.5 mg of acetone, ethanol, protein (BSA), Cu(II), Mn(II), Zn(II), Pb(II), Ni(II), and 0.1 mg of Hg(II).

Analysis of Samples

The determination of nucleic acids in samples prepared in the Experimental section was carried out. The recovery of DNA was between 93.0% and 116% and that of RNA between 95.5% and 111%.

CONCLUSION

The investigation to the interactions of PLV and NR with nucleic acids by visible spectrophotometry supports the Langmuir monolayer adsorption and hypothesis of the microelectrostatic field. The MSASC technique cannot give the higher sensitivity than other methods such as RLS[7] but it improves the precision and accuracy of trace analysis and offers the additional benefits of simplicity and versatility. Therefore, the classical spectrophotometry can still play important role in the recognition of biological macromolecule.

ABBREVIATIONS

ctDNA	Calf thymus deoxyribonucleic acid
DNA	Deoxyribonucleic acid
MSASC	Microsurface-spectral correction technique
RSD	Relative standard deviations

PLV	Proflavine
NR	Naphthol red
yRNA	Yeast ribonucleic acid

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