Interactions of Night Blue with Nucleic Acids and Determination of Nucleic Acids Using Resonance Light Scattering Technique

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The noncovalent interactions of night blue (NB) with several nucleic acids in buffer medium of Britton-Robinson at pH 4.1 have been studied by spectroscopic methods. It is shown that the binding of NB with nucleic acids involves the *J*-aggregation of NB molecules on the surface of nucleic acids. The aggregation was encouraged by polyanions nucleic acids, in which nucleic acids served for acting templates. In this connection, a new method of nucleic acids with sensitivity at nanogram level is proposed based on the measurement of enhanced resonance light scattering (RLS). The linear range of ctDNA, fsDNA and yRNA is 0.01—2.5, 0.03—2.5 and 0.04—1.0 μg/mL, respectively, and the corresponding detection limits (3σ) are 9.4, 7.3 and 5.7 ng/mL at 2.5×10^{-5} mol/L of NB. Synthetic and real samples were analyzed with satisfactory results.

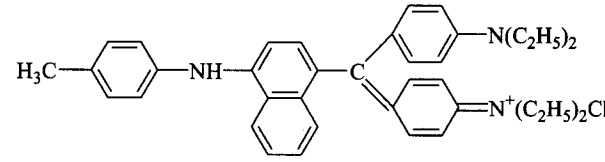
Keywords night blue, nucleic acids, resonance light scattering

Introduction

Considerable studies have been made on a relationship between the three-dimensional structure of DNA and fluorescent probes. Many of these studies are related to the development of fluorescent probes for specific sequences of biopolymer nucleic acids. By altering the photoreactivity of the fluorophore, it is likely to initiate photochemical reactions of the probe with the DNA backbone for DNA strand cleavage, and such specific site photochemical DNA cleavers are significant in gene manipulation and biotechnology. More recently, laser facilities are employed to detect single DNA molecule and the growth of DNA in a polymerase chain reaction (PCR) test based on the fluorescence enhancement effect of DNA on laser dye.[1-3]

On the other hand, resonance light scattering (RLS) technique as another promising tool has attracted increasing interest from chemists and biochemists due to high sensitivity in the characterization of the reactions of organic dyes that can undergo upon exposure to nucleic acids.[4-10] In this paper, the binding interaction of nucleic acids with an easy available dye, night blue (NB) (Scheme 1), which has not long been paid too much attention, was studied. It is shown that the binding of NB with nucleic acids involves the *J*-aggregation of NB molecules on the surface of nucleic acids. The findings of binding interaction of NB with nucleic acids are likely to contribute to design new probe analogs of the compound. Besides, that we report here is also related to the application of NB as probe for nucleic acids assay based on enhanced RLS signal, which leads to a particularly stable, rapid and simple method, permitting a low detection limit (3σ) for nucleic acids. This NB method is much steadier than most of the reported RLS methods.[6,8] To date, many dyes have been proposed for nucleic acids determination. The most widely used dye ethidium bromide (EB) has been considered as a strong carcinogenic compound.[11] Some dimeric dyes, such as ethidium homodimer (EthD),[12] thiazole orange homodimer (TOTO),[13] and oxazole yellow homodimer (YOYO),[14] offer high sensitivity. However, the widespread use of these methods is restricted by the high cost of the dyes. Therefore, for many years, researchers have made great efforts to develop proper dyes for determination of nucleic acids. This NB method is much more sensitive than some dye- and complex-binding methods,[15-18] and is comparable with EB assay in sensitivity.[11] Moreover, it is inexpensive, convenient and applicable. This high sensitivity of RLS technique suggests that the experimental approach can be expanded to the applications in immunochemistry genetic diagnosis and PCR for the *in vitro* exponential amplification of specific nucleic acid sequence.

Scheme 1 Structure of night blue (NB)



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Experimental

Materials

Calf thymus DNA (ctDNA) and yeast RNA (yRNA) were obtained from Sino-American Biotechnology Company. Fish sperm (fsDNA) was purchased from Shanghai Institute of Biochemistry. Nucleic acids were directly dissolved in water and stored at 4 ℃. Their concentrations were determined spectrophotometrically[19,20] by using the following extinction coefficients at 260 nm: DNA, 0.020 $mL \cdot \mu g^{-1} \cdot cm^{-1}$ or 6600 $L \cdot mol^{-1} \cdot cm^{-1}$; yRNA, 0.024 $mL \cdot \mu g^{-1} \cdot cm^{-1}$ or 7800 $L \cdot mol^{-1} \cdot cm^{-1}$. Night blue was obtained from Merck Company and its working solution (5.0×10^{-5} mol/L) was prepared by dissolving the crystals in 40% ethanol without further purification. Hoechst 33258 and ethidium bromide were obtained from Aldrich Chemical Company and Sino-American Biotechnology Company, respectively. Sodium dodecyl benzene sulfonate (SDBS) was purchased from Fluka. Organic polyanions sodium polyacrylate was kindly offered by Polymer Chemistry Laboratory of Nankai University. Britton-Robinson buffer solution was used to control the acidity of the interacting system, while 2 mol/L NaCl solution was used to adjust the ionic strength of the test solutions.

All other chemicals were of analytical reagent grade or better and the deionized distilled water was used throughout.

Apparatus

A Shimadzu RF-540 Spectrofluorometer (Kyoto, Japan) was used to scan the RLS spectra and the fluorescence spectra, and to measure the RLS intensities and the polarizations. A Shimadzu UV-240 Ultraviolet-Visible Spectrophotometer (Kyoto, Japan) was used to obtain the absorption spectra, and to record the absorbance. A pHS-2C pH meter (Shanghai, China) was used to measure the pH values of the interacting systems.

Procedure

Buffer solution (1 mL), 5 mL of NB working solution, and an appropriate volume of nucleic acids, or a sample solution containing nucleic acids were added to a 10-mL flask (V type). The mixture was stirred thoroughly before being diluted to 10 mL with water.

RLS spectra were obtained by scanning synchronously with the same excitation and emission wavelengths. The RLS intensities were measured by keeping wavelengths of excitation and emission at 400 nm. The enhanced RLS intensity of NB-nucleic acids system was represented as $\Delta I = I - I_0$, where I and I_0 were the RLS intensities of NB with and without nucleic acids, respectively.

The fluorescence polarization measurements were made on a Shimadzu RF-540 Spectrofluorometer with a pair of polarizers. The solutions were excited at 540 nm and the fluorescence signal was monitored at 590 nm, through crossed polarizers.

Results and discussion

Resonance light scattering spectra

The interaction of cation agent with nucleic acids is known to induce aggregated products that scattering light.[21-24] Fig. 1 illustrates the increase in RLS during titration of NB with ctDNA. At a ratio of NB to phosphate of ctDNA (R) below 3.3, the solution turned cloudy with a drop due to the on-set of precipitation of complex. Similar RLS spectra were also found for the interaction of NB with fsDNA and yRNA. For the sake of avoiding both precipitation of the nucleic acids and complexities in interpretation of observed data, all further studies therefore were done at R value above 3.3 for ctDNA and fsDNA, 3.25 for yRNA, respectively. The similarity seen in R among nucleic acids indicates that the precipitation behaviour is dependent on charge ratio rather than length/size of nucleic acids.[22]

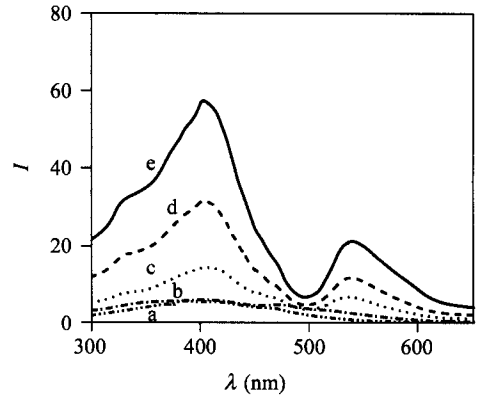

Fig. 1 RLS spectra of NB (a), ctDNA (b) and NB-ctDNA (c to e). NB: 2.5×10^{-5} mol/L but (a) without any NB; ctDNA (μg/mL): (a) 0.0, (b) 3.0, (c) 0.4, (d) 1.2, (e) 2.4, pH: 4.1.

According to the theory of RLS,[4,25-27] the enhanced RLS features are associated with the molecular absorption. Usually the enhanced RLS appears at the red side of absorption band of the colored solution.[25,26,28] Hence, the RLS peak at 400 nm is ascribed to the molecular absorption band of NB in the range of 280—400 nm (Fig. 2). Because the enhancement of RLS is closely related to the degree of electronic coupling among the chromophore and the size of aggregates,[4,5,29] the new RLS peak appeared at 540 nm is probably related to the intense electrostatic attraction between positively charged NB and negatively charged macromolecules.[24] Fig. 3 showed that NB-ctDNA, NB-fsDNA, NB-yRNA, NB-SDBS and NB-sodium polyacrylate have similar RLS spectra. The observations can be reasonably assigned to dye-nucleic acid complex salts based on the electrostatic and hydrophobic interactions between NB and nucleic acids.

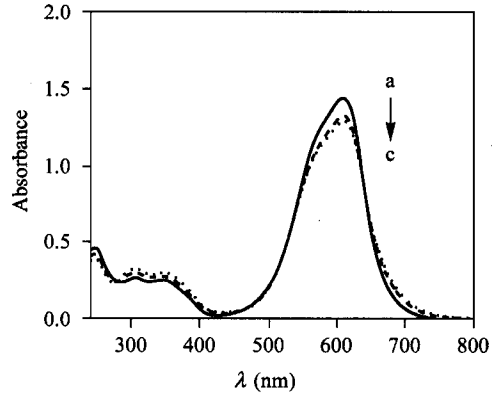

Fig. 2 Absorption spectra of the interacting system of NB with ctDNA. NB: 2.5×10^{-5} mol/L; ctDNA (in decreasing order at 612 nm, μg/mL): (a) 0.0, (b) 2.0, (c) 2.5; pH: 4.1.

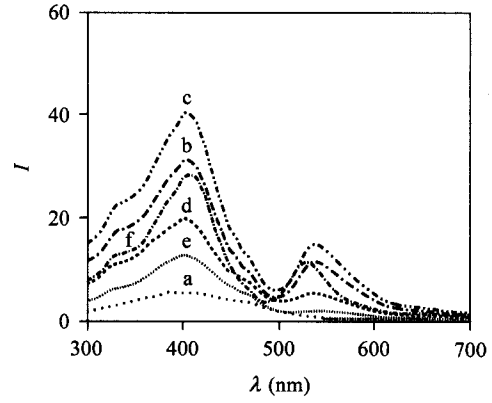

Fig. 3 RLS spectra of NB (a), NB-ctDNA (b), NB-fsDNA (c), NB-yRNA (d), NB-SDBS (e) and NB-sodium polycrylate (f). NB: 2.5×10^{-5} mol/L; ctDNA: 1.2 μg/mL; fsDNA: 1.2 μg/mL; yRNA: 0.4 μg/mL; SDBS: 8.0×10^{-7} mol/L; sodium polycrylate: 1.0×10^{-7} mol/L; pH: 4.1.

Features of NB *aggregation*

It was found that the absorbance ratios of 350 nm vs. 250 nm and 306 nm vs. 250 nm increased with the increase of NB concentration (Table 1), which shows NB has self-aggregation tendency in the test medium.[24] In another words, the self-aggregation is encouraged with the increase of NB concentration. It is inferred that the absorption band in the range of 280—400 nm should originate from the aggregation of the species, which are associated with the absorption band at 250 nm (Fig. 2). On the basis of aggregation concept, the 280—400 nm band should be ascribed to the *J*-aggregation of 250 nm band since the band of aggregation locates on the red side of 250 nm (Fig. 2).[7,30]

Table 1 Absorbance ratios of the characteristic bands of NB

NB concentration (10^{-5} mol/L)	A_{350}(nm)/A_{250}(nm)	A_{306}(nm)/A_{250}(nm)
0.3	0.500	0.600
0.5	0.536	0.607
1.0	0.540	0.613
1.5	0.558	0.617
2.0	0.569	0.625
2.5	0.578	0.630
3.0	0.586	0.635
3.5	0.595	0.643
4.0	0.669	0.655

Study of binding site

In order to locate the possible binding site of NB on DNA, fluorescence experiments were completed by using minor groove binding agent Hoechst 33258[31] as reporter molecule. The titration of NB into DNA-Hoechst complex led to an obvious decrease of fluorescence intensity as well as a significant blue shift for either excitation or emission spectra up to a NB concentration of 1.0×10^{-6} mol/L, beyond which a rapid rise resulted without further shifts of peak positions due to disassociation of the minor groove probe[21] (Fig. 4). Given that, NB could compete with Hoechst 33258 for the minor groove site in DNA, the first addition of NB would be expected to result in an increase instead of a decrease in fluorescence intensity based on the ability of DNA that quenches the fluorescence of Hoechst 33258. Consequently, it is inferred that the addition of NB to DNA-Hoechst complex did not result in displacement of the probe, but the observable fluorescence (excitation and emission maxima) of Hoechst 33258 changed significantly, reflecting an alteration in Hoechst 33258 environment. This behaviour is characteristicaly elicited by intercalator EB, and hence fluorescence polarization experiment was carried out with EB. Fig. 5 showed the effect of increasing NB concentration on the polarization of EB bound to DNA. It should be noted that an intercalatively bound form of EB, held more rigidly in the helix with long residence times on the time scale of the emission lifetimes, should yield finite polarization, while free EB would not contribute significantly to the polarization based on the rapid tumbling motion of the EB molecule in the test media.[32,33] Therefore, if NB could intercalate into the helix of DNA, addition of NB would be expected to produce a decrease in polarization

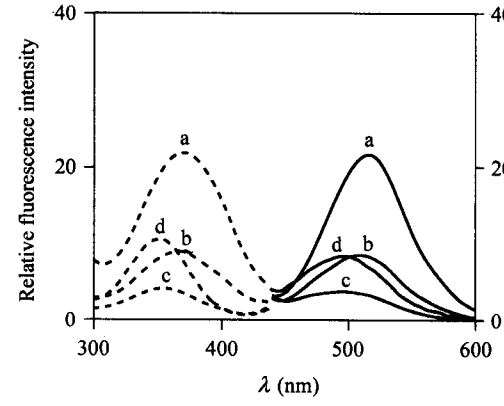

Fig. 4 NB effect on the fluorescence excitation (---) and emission (—) spectra of Hoechst-ctDNA system. Hoechst: 4.0×10^{-7} mol/L; ctDNA: 0.53 μg/mL; NB ($\times 10^{-6}$ mol/L): (a) 0.0, (b) 0.5, (c) 1.0, (d) 1.5; pH: 4.1.

of EB-DNA complex system due to the displacement of the bound EB molecules. In fact, as shown in Fig. 5, the polarization of the system generally keeps constant with increasing NB to EB-DNA complex. In other words, the displacement of the probe EB does not occur. On all accounts, the results of experiments suggest that the NB molecules should bind DNA from the surface of DNA duplex or at least partly from the minor groove side, since NB disintercalates EB and changes the environment around Hoechst 33258 bound in the minor groove.[21]

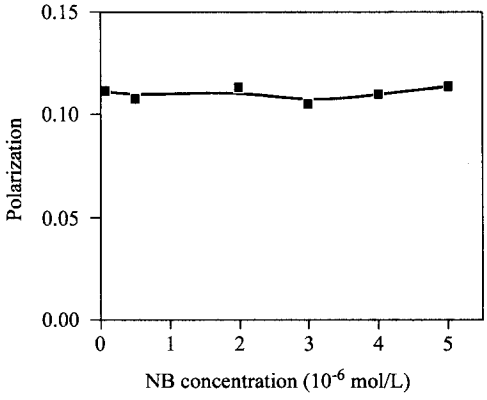

Fig. 5 Competitve binding between NB and EB for ctDNA. ctDNA: 0.82 μg/mL; EB: 5 μg/mL; pH: 4.1; λ_{ex}: 540 nm; λ_{em}: 590 nm.

Effect of pH

The effects of pH on the RLS intensity of NB-ctDNA system are shown in Fig. 6. The enhanced RLS intensity (ΔI) is stable in the range of 3.7—4.5, and other pH range produces a small value of ΔI. pH 4.1 was chosen for the assay.

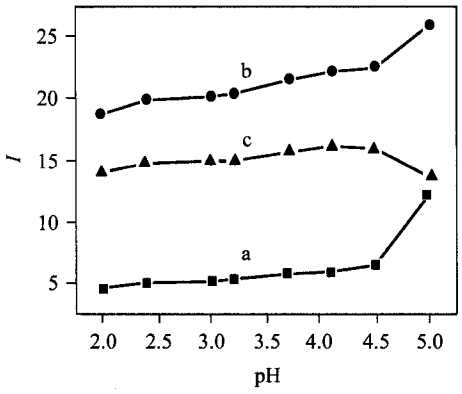

Fig. 6 pH effect on the RLS response of NB (a), NB-ctDNA (b) and the difference between NB-ctDNA and NB (c). NB: 2.5×10^{-5} mol/L; ctDNA: 0.75 μg/mL; λ: 400 nm.

Effect of solvent and NaCl

The effects of ethanol and NaCl were examined either in the absence or in the presence of ctDNA. When the concentration of the ethanol reaches 20%, the enhanced RLS signal goes up to the maximum (Fig. 7). A continuous increase in ethanol content was followed by decrease in the RLS intensity. This could be because ethanol changed the refractive index of interaction system.[34] Alternatively, when NaCl concentration is < 0.008 mol/L, the RLS intensities remain constant. Once more NaCl was added into the solution, the enhanced RLS intensity (ΔI) would clearly become small with the increase of NaCl concentration.

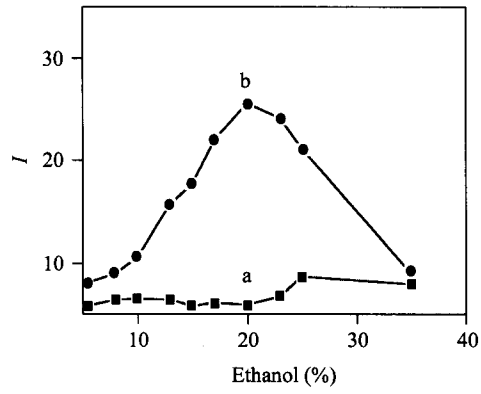

Fig. 7 Effect of ethanol on the intensity of RLS of NB without (a) and with (b) ctDNA. NB: 2.5×10^{-5} mol/L; ctDNA: 0.9 μg/mL; pH: 4.1; λ: 400 nm.

Stability

The test showed that the reaction between NB and nucleic acids is immediate at room temperature (< 1 min), and the scattering intensity is stable for at least 7 h and is not affected by the addition sequence.

Effect of foreign substances

The interference of various ions, nucleotides, amino acid, sugars, surfactants, vitamin and BSA was tested at Britton-Robinson buffer medium (pH 4.1) (Table 2). It was found that these foreign substances had little effects on the determination of nucleic acids under the permission of 5% error.

Table 2 Tolerance of foreign substances[a]

Substance	Concentration (10^{-6} mol/L)	Change of ΔI (%)
Na(I) chloride	22000	+3.1
Ca(II) chloride	15	-6.2
Zn(II) nitrate	15	-4.9
Al(III) nitrate	25	0.0
Mg(II) chloride	35	-7.4
Cd(II) nitrate	30	0.0
Pb(II) nitrate	0.5	+2.0
Mn(II) sulfate	15	-4.4
Ni(II) chloride	5	-4.9
Cu(II) nitrate	10	-5.7

Continued

Substance	Concentration (10^{-6} mol/L)	Change of ΔI (%)
Co(II) chloride	25	0.0
Fe(III) nitrate	0.9	+8.3
Cr(III) nitrate	40	-4.7
Hg(II) nitrate	10	-4.8
β-CD	7	-4.5
SDS	0.4	+3.6
SLS	6	+6.1
Triton X-100[b]	0.02	+5.2
CTMAB	5	0.0
ADP	1.7	-5.7
AMP	4	-1.1
GMP	6	-5.5
BSA[c]	0.5	+5.5
Histidine	5	-5.0
Lysine	45	-2.5
Glycine	50	-2.0
Alanine	50	+3.3
Glucose	50	-3.3
Sucrose	50	-4.4
Lactose	55	-3.3
Ascorbic acid	25	+4.8

[a] NB: 2.5×10^{-5} mol/L; ctDNA: 1.2 μg/mL; pH: 4.1; λ: 400 nm. [b] Represented by %. [c] Represented by μg/mL.

Analytical application

Under the optimum conditions, a linear relationship for an appropriate NB solution was established between ΔI and the concentration of nucleic acids. All of the analytical parameters are presented in Table 3. It can be found that NB concentration affects the linear range and the sensitivity of the determination. So, an appropriate range can be arranged by using suitable concentrations of NB, and then the determination of nucleic acids can be carried out accordingly.

The proposed method was applied to the determination of nucleic acids in the synthetic samples prepared based on the interference of foreign substances (Table 2). The analytical results are presented in Table 4. Moreover, DNA extracts from spartina anglica, which were obtained from Biochemistry Department of Nankai University, were analyzed using the proposed method and the ethidium bromide fluorometry.[11] The analytical results by the two methods are very closed with satisfactory reproducibility (Table 5). It clearly indicates that the proposed method is reliable, sensitive, simple and practicable.

Conclusion

The enhanced RLS signal originates from aggregation effect of NB on the surface of nucleic acids. Similar phenomena were also observed by the reaction of NB with SDBS and sodium polyacrylate, which indicates NB molecule reacts with nucleic acids mainly by electrostatic and hydrophobic forces. Further evidence from fluorescence experiments elicits the possible binding site of NB on DNA. NB molecules bind DNA

Table 3 Analytical parameters for the determination of nucleic acids[a]

Nucleic acid	Concentration of NB (10^{-5} mol/L)	Linear range (μg/mL)	Equation (C, μg/mL)	DL[b] (ng/mL)	Correlation coefficient
ctDNA	2.0	0.01—2.0	$\Delta I = -0.3 + 20.9C$	9.6	0.9989 ($n = 6$)
	2.5	0.01—2.5	$\Delta I = 0.2 + 21.2C$	9.4	0.9994 ($n = 7$)
	3.0	0.01—3.0	$\Delta I = -0.4 + 18.9C$	10.6	0.9984 ($n = 8$)
fsDNA	2.0	0.03—2.0	$\Delta I = -0.1 + 25.6C$	7.8	0.9989 ($n = 6$)
	2.5	0.03—2.5	$\Delta I = -0.5 + 27.4C$	7.3	0.9993 ($n = 7$)
	3.0	0.03—3.0	$\Delta I = 0.3 + 26.6C$	7.5	0.9991 ($n = 8$)
yRNA	2.5	0.04—1.0	$\Delta I = 0.3 + 35.0C$	5.7	0.9995 ($n = 5$)

[a] In pH 4.1 Britton-Robinson buffer medium, all data were obtained at 400 nm. [b] Detection limit.

Table 4 Analytical results of nucleic acids in the presence of some additives ($n = 5$)[a]

Nucleic acid in samples (μg/mL)	Main additives[b]	Found value (μg/mL)	Recovery (%)	RSD[c] (%)
ctDNA 2.00	BSA, Ca^{2+}, Zn^{2+}, Mg^{2+}	2.01	98.8—101.2	1.3
ctDNA 1.20	AMP, ADP, GMP, Al^{3+}	1.22	99.5—105.3	2.4
fsDNA 2.00	BSA, glucose, Zn^{2+}, Cr^{3+}	2.05	100.7—104.3	1.4
fsDNA 1.20	AMP, ADP, GMP, alanine	1.21	96.6—102.2	2.3
yRNA 0.60	AMP, ADP, GMP, ascorbic acid	0.58	92.0—100.4	3.6
yRNA 1.00	BSA, lactose, Cu^{2+}, Zn^{2+}	0.98	95.1—105.4	4.4

[a] NB: 2.5×10^{-5} mol/L; pH: 4.1; λ: 400 nm. [b] BSA: 0.1 μg/mL; AMP, ADP or GMP: 1.0×10^{-6} mol/L; Ca^{2+}, Zn^{2+}, Mg^{2+}, Al^{3+}, Cr^{3+}, Cu^{2+}, glucose, alanine, lactose or ascorbic acid: 2.5×10^{-6} mol/L. [c] Relative standard deviation for five measurements.

Table 5 Determination results for DNA extracts ($n = 3$)[a]

	This method	RSD (%)	Ethidium assay[11]	RSD (%)
	Mean (g/L)		Mean (g/L)	
Spartina anglica DNA1	0.492	3.9	0.478	3.8
Spartina anglica DNA2	0.484	3.2	0.475	3.6

[a] NB: 2.5×10^{-5} mol/L; pH: 4.1; λ: 400 nm.

duplex from the surface of DNA or at least partly from the minor groove side since NB disintercalates EB and changes the environment around Hoechst 33258 bound in the minor groove. It was found that the enhanced RLS signal is proportional to the concentration of nucleic acids, and the determinations of nucleic acids at ng level can be carried out. The proposed method is featured by its low detection limit, rapid reaction, good stability and relative simplicity.

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