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The Sensitive Determination of Nucleic Acids Using Fluorescence Enhancement of Eu^{3+}-Benzoylacetone-Cetyltrimethylammonium Bromide-Nucleic Acid System

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A new quantitative method for micro amounts of nucleic acids in aqueous solution is proposed using Eu^{3+}-benzoylacetone (BA) complex as fluorescent probe in the presence of cetyltrimethylammonium bromide (CTMAB). Under the optimum condition, the ratio of the fluorescence intensities with and without nucleic acids is proportional to the concentration of nucleic acid in the range of 1.0×10^{-9} to 5.0×10^{-6} g/mL for herring sperm DNA (hsDNA), 3.0×10^{-9} to 1.0×10^{-6} g/mL for calf thymus DNA(ctDNA) and 8.0×10^{-9} to 1.0×10^{-6} g/mL for yeast RNA (yRNA), and their detection limits are 0.33, 0.21 and 0.99 ng/mL, respectively. Actual sample (DNA of *Arabidopsis thaliana*) was determined satisfactorily. In addition, the interaction mechanism is also investigated.

KEY WORDS: Fluorescence; nucleic acids; Eu^{3+}-Benzoylacetone(BA)-CTMAB.

INTRODUCTION

Nucleic acids have an important function in life process, so the study on them is important for the field of Life Science. The quantitative analysis of nucleic acids can be used in many fields such as biochemistry, molecular biology, biotechnology, and medical diagnostics. Fluorescence method has high sensitivity, so it is often used for quantitative analysis of micro amounts of nucleic acids. But the direct use of the natural fluorescence emission properties of nucleic acids for their structural and quantitative analysis has been limited [1,2] due to the low fluorescence quantum yield of native DNA ($\Phi_f = 4 \times 10^{-5}$) [3], so extrinsic probes must be employed. Generally, the studies of fluorescent probes for the determination of nucleic acids focuses on organic dyes [3–7], rare-earth ions [8–10], metal complexes [11–13], etc. Especially, rare-earth complexes are widely applied because they not only have the rare-earth luminescence characteristics such as narrow spectral width, long luminescence life-time, large stocks shift, but also have higher sensitivity than rare-earth ions probes. Reported ligands of rare-earth ions have phenanthroline [14], tetracycline [15], 8-hydroxyquinoline [16], tiron [17], 2-oxo-4-hydroxy-quinoline- 3-carboxylic acid [18] and, so on.

It is well known that β-diketone can chelate with lanthanide ions and sensitize fluorescence of rare-earth ions (such as: Eu^{3+}, Sm^{3+}, Tb^{3+}, Dy^{3+}) [19–21]. In this paper, BA (β-diketone) is selected as a ligand. The Eu^{3+}-BA-CTMAB ion association system exhibits a low level characteristic fluorescence of Eu^{3+}, whereas the fluorescence intensity of the system is considerably enhanced by nucleic acids. Based on this phenomenon, the novel method for determination of nucleic acids concentration is developed.

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EXPERIMENTAL

Chemicals

Stock standard solution (1.00×10^{-2} mol/L) of Eu^{3+} was prepared by dissolving Eu_2O_3 (Yuelong Chemical Co. Shanghai, 99.9%) in hydrochloric acid, evaporating to near dryness and diluting with 0.1 mol/L HCl.

Benzoylacetone solution (1.00×10^{-2} mol/L) was prepared by dissolving 0.0810 g of BA in 50 mL volumetric flask with 95% alcohol.

Stock solutions of nucleic acids (100 μg/mL) were prepared by dissolving commercially available herring sperm DNA (hsDNA, Sigma, Germany), calf thymus DNA (ctDNA, Sigma, Germany) and yeast RNA (yRNA, Sigma, Germany) in 0.05 mmol/L sodium chloride solution. These stocks were stored at $0 \sim 4^{\circ}$C in refrigerator. Working solutions were prepared by appropriate dilution with water.

Stock solution of cetyltrimethylammonium bromide (CTMAB, 1.0×10^{-3} mol/L) was prepared by dissolving 0.1822 g of CTMAB in 500 mL water.

A 0.05 mol/L Tris-HCl buffer solution was prepared by dissolving 3.030 g Tris in 500 mL deionized water and then the pH was adjusted to 8.25 with HCl, using an acidity meter.

All the chemicals used were of analytical grade and doubly deionized water was used throughout.

Apparatus

The fluorescence spectra and the intensity were recorded with a LS-55 spectrofluorimeter (Perkin–Elmer Corp., USA). The surface tension was measured on Processor Tensiometer-K12 (Krüss Corp) with the precise degree of the measurement 0.01 mNm^{-1} by the Wilhelmyplate. All pH measurements were made with a Delta 320-S acidity meter (Mettler Toledo, Shanghai).

Procedure

To a 25-mL test tube, solutions were added in the following order: Eu^{3+}, BA, CTMAB, DNA (or RNA), Tris-HCl buffer. The mixture was diluted to 10 mL with deionized water and shaken. After a standing time of 20 min at room temperature, the fluorescence intensity was measured in a 1-cm quartz cell, the excitation and emission slits were both 10 nm with a scan speed of 500 nm/min. The enhanced fluorescence intensity of Eu^{3+}-BA-CTMAB by nucleic acids was represented as I_f/I_0. Here, I_f and I_0 are the intensities of the systems with and without nucleic acid, respectively.

RESULT AND DISCUSSION

Fluorescence Spectra

The excitation and emission spectra of Eu^{3+}-BA (1), Eu^{3+}-BA-hsDNA (2), Eu^{3+}-BA-CTMAB (3), Eu^{3+}-BA-CTMAB-yRNA (4), Eu^{3+}-BA-CTMAB-ctDNA (5) Eu^{3+}-BA-CTMAB-hsDNA (6) systems are shown in Fig. 1. From this figure, it can be seen that all systems have the same excitation peak at 350 nm, and two emission peaks at 592 nm and 615 nm, corresponding to the

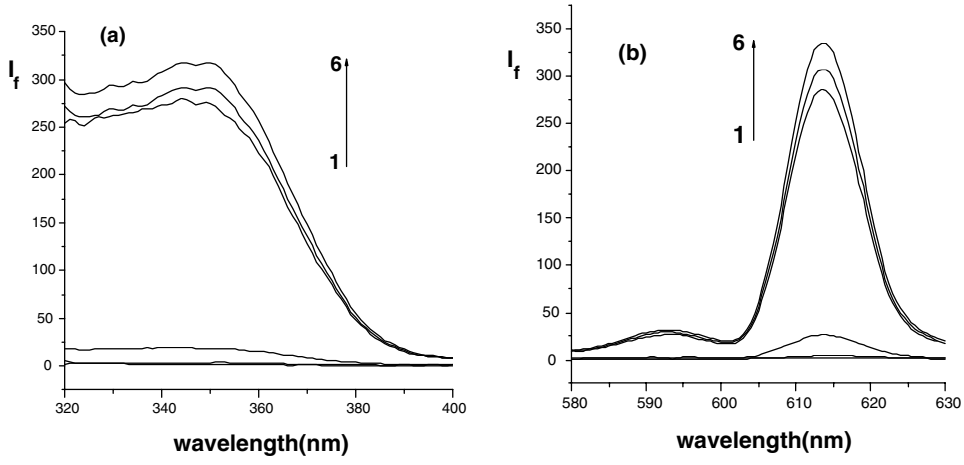

Fig. 1. (a) Excitation and (b) emission spectra. 1. Eu-BA-Tris; 2. Eu-BA-hsDNA-Tris; 3. Eu-BA-CTMAB-Tris; 4. Eu-BA-CTMAB-yRNA-Tris; 5. Eu-BA-CTMAB-ctDNA-Tris; 6. Eu-BA-CTMAB-hsDNA-Tris. Conditions: Eu^{3+}: 5.0×10^{-6} mol/L, BA: 8.0×10^{-5} mol/L, CTMAB: 5.0×10^{-5} mol/L, hsDNA: 1.0×10^{-6} g/mL, yRNA: 1.0×10^{-6} g/mL,Tris-HCl 5.0×10^{-3} mol/L (pH 8.25).

$^5D_0 \rightarrow {}^7F_1$ and $^5D_0 \rightarrow {}^7F_2$ transitions of Eu^{3+}, respectively. Although the fluorescence intensity of Eu-BA system can be enhanced by CTMAB, its intensity is weak. However, after the addition of nucleic acid, the fluorescence intensity of the system is greatly enhanced, which indicates that there is the interaction between nucleic acid and Eu^{3+}-BA–CTMAB complex. The fluorescence intensity at 615 nm is the strongest, so 615 nm was chosen as the emission wavelength in further research.

Effect of pH and Buffers

The effect of pH on the fluorescence intensities ratio of the assay systems with (I_f) and without (I_0) nucleic acids was studied. The experimental results indicate that the fluorescence intensity ratio I_f/I_0 reaches the maximum in the pH range of 8.2–8.5 (Fig. 2). Different buffers: HMTA, Tris-HCl, $Na_2B_4O_7$-HCl, NH_4Cl-NH_3, NaAc-HAc, and $Na_2B_4O_7$-H_3BO_3 were tested at pH 8.3 ± 0.1. The results indicate that the I_f/I_0 is the largest in Tris-HCl buffer. Further research proves that 1.0 mL of Tris-HCl buffer at pH 8.25 is appropriate. In order to improve the sensitivity and selectivity, 1.0 mL 0.05 mol/L Tris-HCl is used in this assay.

Effect of Eu^{3+} Concentration

The effect of the concentration of Eu^{3+} was tested. The fluorescence intensity ratio (I_f/I_0) curve versus Eu^{3+} concentration is shown in Fig. 3. It can be seen that the I_f/I_0 increases with the increase of Eu^{3+} concentration when the Eu^{3+} concentration is less than 4.0×10^{-6} mol/l, and is almost constant at the range of $4.0 \times 10^{-6}$to 6.0×10^{-6} mol/l. After that the I_f/I_0 decreases obviously. So 5.0×10^{-6} mol/L of Eu^{3+} is selected for further research.

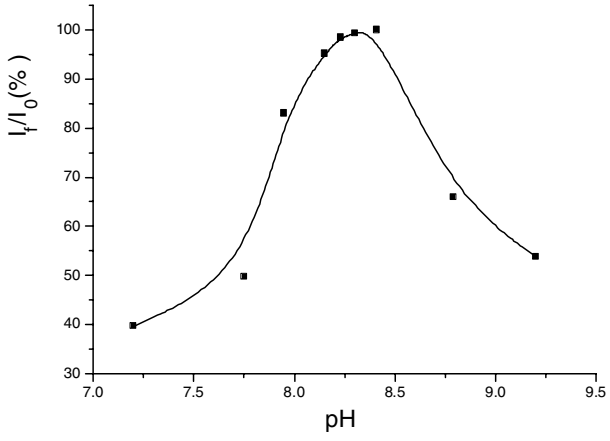

Fig. 2. Effect of pH. Conditions: Eu^{3+}:5.0×10^{-6} mol/L, BA: 8.0×10^{-5} mol/L, CTMAB: 5.0×10^{-5} mol/L, hsDNA: 1.0×10^{-6} g/mL.

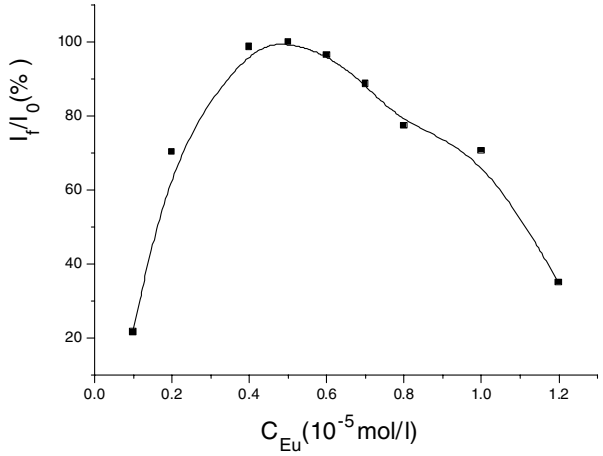

Fig. 3. Effect of Eu^{3+} concentration. Conditions: Eu^{3+}: 5.0×10^{-6} mol/L, BA: 8.0×10^{-5} mol/L, CTMAB: 5.0×10^{-5} mol/L, hsDNA: 1.0×10^{-6} g/mL, Tris-HCl 5.0×10^{-3} mol/L (pH 8.25).

Effect of BA Concentration

The effect of BA concentration was studied as shown in Fig. 4. It is found that the I_f/I_0 of the Eu-BA-CTMAB-nucleic system reached the maximum when the concentration of BA was 8.0×10^{-5} mol/L under the given conditions. Therefore, 8.0×10^{-5} mol/L BA is chosen for this assay.

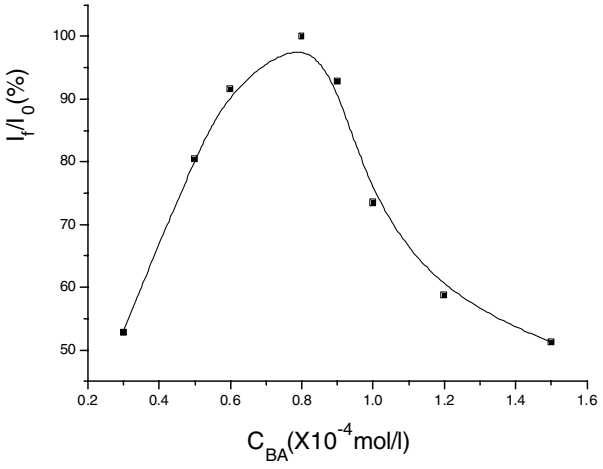

Fig. 4. Effect of BA concentration. Conditions: Eu^{3+}: 5.0×10^{-6} mol/L, BA: 8.0×10^{-5} mol/L, CTMAB: 5.0×10^{-5} mol/L, hsDNA: 1.0×10^{-6} g/mL, Tris-HCl 5.0×10^{-3} mol/L (pH 8.25).

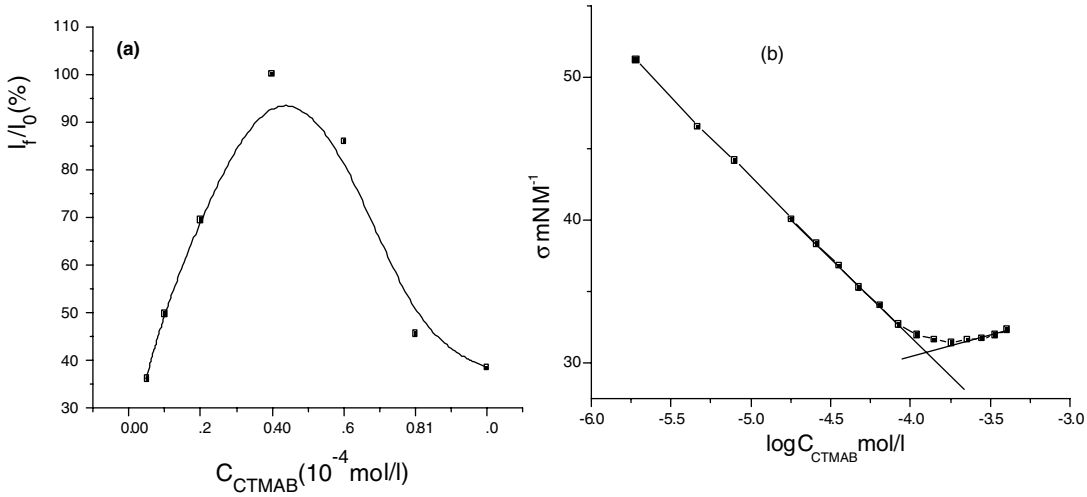

Fig. 5. (a) Effect of CTMAB concentration (b) and surface tension of CTMAB. Conditions: Eu^{3+} 5.0×10^{-6} mol/L, BA 8.0×10^{-5} mol/L, hsDNA 10×10^{-6} g/mL, Tris-HCl 5.0×10^{-3} mol/L (pH 8.25).

Effect of Surfactants

The effect of following surfactant on the I_f/I_0 was tested: CTMAB, cetylpyridine bromide (CPB), sodium dodecyl sulphonate (SDS), sodium dodecyl benzene sulphonate (SDBS), sodium dodecyl sulphate (SLS), and OP. The results show that only CTMAB has remarkable enhancement effect on the system.

The effect of CTMAB concentration on the I_f/I_0 was studied as shown in Fig. 5a. It can be seen that the CTMAB concentration affects the I_f/I_0 seriously. The I_f/I_0 of the system increases with the increase of CTMAB concentration and reaches a maximum at 5.0×10^{-5} mol/L CTMAB, and then decreases obviously. Therefore, 5.0×10^{-5} mol/L is selected in the research.

The plot of the surface tension versus logarithm of the CTMAB concentration is shown in Fig. 5b. The CMC value was estimated from the intersection of the extrapolated linear portions of the plot of surface tension versus logarithm of CTMAB concentration, the concentration 1.2×10^{-4} mol/L might be regarded as the apparent critical micelle concentration (CMC) of CTMAB in this system. The results showed that the selected concentration of CTMAB is below its CMC, which shows that CTMAB exists as the pre-micelle or individual monomers in the studied system.

Effect of Addition Order of the Reagents and Incubation Time

The addition sequence of reagents affects the fluorescence intensity ratio (I_f/I_0) of the system. Detailed researches indicate that the addition in the order of Eu^{3+}, BA, CTMAB, nucleic acid and Tris-HCl is the best.

Under the optimum condition, the effect of reaction time on the I_f/I_0 was investigated. The results showed that the determination signal reaches a maximum 20 min after all the reagents are added and remains stable for over 3 h.

Effect of Foreign Substance

The interference of foreign substances was tested according to the standard procedure. The tolerance limit is taken under the permission of ±5% relative error. As shown in Table I, most of the inorganic ions have little interference in the assay.

Table I. Effect of Foreign Substances

Substances	Concentration ($\times 10^{-6}$ mol/L)	Change of I_f (%)
K^+, Cl^-	5.0	+3.37
Ca^{2+}, Cl^-	12.5	+4.62
Mn^{2+}, SO_4^{2-}	3.0	+6.99
Ba^{2+}, Cl^-	5.0	+5.22
Al^{3+}, NO_3^-	3.0	+1.13
Na^+, Cl^-	5.0	+3.6
Mg^{2+}, SO_4^{2-}	5.0	+3.06
Al^{3+}, Cl^-	3.0	+2.73
Na^+, CO_3^{2-}	3.0	+5.98

Note. All the values were obtained by the standard procedure the conditions: Eu^{3+}: 5.0×10^{-6} mol/L, BA: 8.0×10^{-5} mol/L, CTMAB: 5.0×10^{-5} mol/L, hsDNA: 1.0×10^{-7} g/mL,Tris-HCl 5.0×10^{-3} mol/L (pH 8.25).

Table II. Analytical Parameters of This Method

Nucleic acid	Linear range (g/mL)	Linear regression equation (μg/mL)	r^a	LOD (g/mL)
hsDNA	1.0×10^{-9}–5.0×10^{-6}	$I_f/I_0 = 1.15 + 2.49C$	0.9977	3.3×10^{-10}
ctDNA	3.0×10^{-9}–1.0×10^{-6}	$I_f/I_0 = 1.17 + 0.87C$	0.9968	2.1×10^{-10}
yRNA	8.0×10^{-9}–1.0×10^{-6}	$I_f/I_0 = 1.49 + 5.85C$	0.9899	9.9×10^{-10}

[a] Correlation coefficient.

Analytical Applications

Under the optimum conditions defined, the calibration curves between the I_f/I_0 and nucleic acids were obtained, and the correlation coefficients reach 0.99 (Table II). It can be seen that there are a linear relationship between the I_f/I_0 and the concentration in the range of 1.0×10^{-9} to 5.0×10^{-6} g/mL for hsDNA, 3.0×10^{-9} to 3.0×10^{-6} g/mL for calf thymus DNA (ctDNA) and 8.0×10^{-9} to 1.0×10^{-6} g/mL for yeast RNA (yRNA), and their detection limits are 3.3×10^{-10} g/mL, 2.1×10^{-10} g/mL, and 9.9×10^{-10} g/mL, respectively. In comparison with other probes (see Table III) of nucleic acids, this probe has higher sensitivity.

Subsequently, an actual sample *Arabidopsis thaliana* was tested by the standard addition method. *Arabidopsis thaliana* is not of major agronomic significance, but it offers important advantages for basic research in genetics and molecular biology fields. The actual sample was isolated from *Arabidopsis thaliana* derived from granular embryogenic calli by using the CTMAB method [22]. The content of DNA in the sample is 1.97 mg/mL, which is obtained by using a Biophotometer (Eppendorf Co.). The sample was diluted 10,000 times and determined by this proposed method, the mean value of the five measurements was 1.98×10^{-7} g/mL and the relative standard deviation was 0.19% ($n = 5$). The sample recovery rate was 94.4–110%. Hence, the proposed method is suitable for the determination of trace amount of nucleic acids in this sample.

Interaction Mechanism of the System

From Fig. 1, it can be seen that when CTMAB and nucleic acid are together added to Eu-BA system, the fluorescence of the system can be greatly enhanced, which indicates that there is the interaction between Eu-BA, CTMAB, and nucleic acid.

From the resonance light scattering (RLS) spectra (Fig. 6), it can be seen that the RLS intensity of nucleic acid-CTMAB system is stronger than those of both nucleic acid and CTMAB, which indicates the formation of an association complex of nucleic acid-CTMAB by electrostatic binding and hydrophobic force [23]. When the Eu-BA complex is added to nucleic acid-CTMAB system, the RLS intensity of this system is greatly enhanced, demonstrating the interaction between Eu-BA and nucleic acid-CTMAB complexes. It is considered that the Eu-BA complex is enwrapped in the CTMAB-nuleic acid association complex by hydrophobic force and electrostatic-attraction to form a larger aggregation. Firstly, the CTMAB-nucleic acid association can provide a hydrophobic microenvironment for the Eu-BA complex, which makes the fluorescence intensity of the latter enhanced. Secondly, BA is excessive for the formation of Eu-BA complex in this system. The abundant BA molecules are also solubilized into nucleic acid-CTMAB cluster by hydrophobic force, which shorten the distance between the Eu^{3+}-BA complex and the excessive BA molecules. So the Eu^{3+} in the Eu^{3+}-BA can accept the energy from the excessive BA molecules through intermolecular energy

Table III. Comparison of the Probe of Detection Limit of Nucleic Acids

Probe	Nucleic acids	LOD (ng/mL)	References
Rivanol	ctDNA/yRNA	62/156	[3]
$Ru(bipy)_2(dppz)^{2+}$	ctDNA/ssDNA/hsDNA	3.3/2.8/4.4	[13]
Tb^{3+}-phenanthroline	ctDNA/fsDNA/yRNA	100/200/200	[14]
Eu^{3+}-Tetracycline	ctDNA/fsDNA	10/10	[15]
Y^{3+}-8-hydroxyquinoline	ctDNA/fsDNA/yRNA	0.03/0.02/.009	[16]
Tb^{3+}-Tiron	ctDNA/smDNA/hsDNA/yRNA	1/1/0.9/0.6	[17]
Tb^{3+}-R_1^a	ctDNA/fsDNA	12/10	[18]
This probe	ctDNA/hsDNA/yRNA	0.21/0.33/0.99	

[a] *N*-[2-(diethylamino)ethyl]-4-hydroxy-2-oxo-1-propyl-1,2-dihydro-3-quniolinecarboxamide.

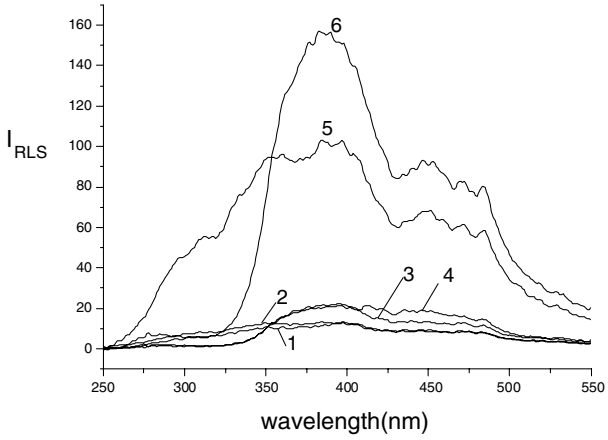

Fig. 6. Resonance light Scattering spectra. 1. hsDNA, 2. CTMAB, 3. Eu^{3+}-BA-hsDNA, 4. Eu^{3+}-BA-CTMAB, 5. CTMAB-hsDNA, 6. Eu^{3+}-BA-CTMAB-hsDNA. Conditions: Eu^{3+}: 5.0×10^{-6} mol/L, BA: 8.0×10^{-5} mol/L, CTMAB: 5.0×10^{-5} mol/L, hsDNA: 1.0×10^{-6} g/mL, Tris-HCl 5.0×10^{-3} mol/L (pH 8.25).

transfer, which also enhances the fluorescence intensity of the system. Consequently, we consider that it is the synergic effect of hydrophobic microenvironment of nucleic acid-CTMAB and the intermolecular energy transfer for the fluorescence enhancement in this system.

CONCLUSIONS

The fluorescence of Eu-BA can be enhanced greatly by the addition of nucleic acids in the presence of proper concentration of CTMAB. Based on this, a novel fluorimetric method has been developed for the determination of nucleic acids and successfully utilized in the actual sample of *Arabidopsis thaliana*. The proposed method has obvious high sensitivity, stability, and reproducibility.

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