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Fluorescence enhancement method for the determination of nucleic acids using cationic cyanine as a fluorescence probe

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A fluorescence enhancement method with a cationic cyanine as a probe was developed for the determination of nucleic acids. Under the experimental conditions, the fluorescence enhancement of cyanine ($\lambda_{ex}/\lambda_{em}$ = 524/591.5 nm) was observed in the presence of DNA. The calibration graphs were linear over the range of 0.01–15 μg mL^{-1} for both calf thymus DNA (CT DNA) and fish sperm DNA (FS DNA). The limits of detection were 0.005 and 0.007 μg mL^{-1} for CT DNA and FS DNA, respectively. The method was applied to the determination of DNA in synthetic and real samples and satisfactory results were obtained. A possible fluorescence enhancement mechanism was also studied.

Introduction

Quantitative determination of nucleic acids is required in many fields, such as molecular biology, biotechnology and medical diagnosis. Using the natural fluorescence of nucleic acids for their structural and dynamic studies and fluorimetric determination has been limited due to the low fluorescence quantum yield (Φ_f = 4 × 10^{-5}) of native DNA,[1] therefore, extrinsic fluorescence probes must be employed. The probes for nucleic acids, which have been reported, include organic dyes such as ethidium bromide,[2] bisimidazole (Hoechst 33258),[3,4] yellow orange dyes,[5] 4,6-diamidino-2-phenylindole-2HCl[6,7] and some trivalent lanthanide cations such as Tb(III)[8] and Eu(III).[9] In addition, some other fluorescent dyes, such as 9,10-anthraquinone-2-sulfonate[10] and Vitamin K_3,[11] have been used as photochemical fluorescence probes for nucleic acid assays. In recent years, some cyanine dyes including dye dimers such as TOTO and YOYO[12,13] have also received much attention.

Current studies of the cyanine dyes have proven their usefulness in numerous analytical application, such as the determination of amino acids,[14] protein,[15,16] amines[17] and toxic drugs,[18] DNA sequencing[19] and detection of micro-environmental changes.[20] Especially, the near-infrared (near-IR) cyanine dyes were characteristic of low background interference, high absorbility, and high fluorescence quantum yield.[21]

We previously synthesized a water-soluble cationic cyanine according to the literature.[22] The cationic cyanine, as a near-IR dye, showed strong fluorescence ($\lambda_{ex}/\lambda_{em}$ = 765/790 nm) in aqueous solution, but the fluorescence was strongly quenched in the presence of nucleic acids. Based on that fact, a fluorescence quenching method for the determination of nucleic acids was developed.[23] Recently, we synthesized a hydrophobic cationic cyanine compound (its structure is shown in Fig. 1) according to ref. 24 and studied its interaction with nucleic acids. The hydrophobic cyanine dye displayed a weak fluorescence emission at 591.5 nm with maximum excitation at 524 nm, however, a significant fluorescence enhancement occurred in the presence of trace amounts of DNA. Furthermore, the enhanced fluorescence intensity was proportional to the concentration of DNA with a good linear relationship under optimum conditions. Therefore, a fluorescence enhancement method was developed for the determination of nucleic acids. In addition to rapid reaction, low detection limit and high stability, the method has a rather wide linear range (beyond three orders of magnitude: 0.01–15 μg mL^{-1} for both calf thymus DNA and fish sperm DNA). Interference experiments show that the assay was scarcely influenced by foreign substances. Recoveries of 96–110.5% were found in the analysis of four synthetic and four real samples. Preliminary research showed that the fluorescence enhancement may be due to the binding of a single cyanine molecule in the minor groove of DNA or the self-assembling of the cyanine dye J-aggregate in the presence of a double-helical DNA template.

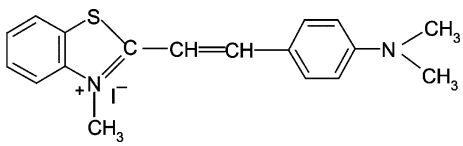

Fig. 1 Chemical structure of the cationic cyanine dye used.

Experimental

Apparatus

A Hitachi F-2500 spectrofluorometer (Tokyo, Japan) equipped with a plotter unit and a 1 cm quartz cell was used for recording fluorescence spectra and making fluorescence measurements. The absorption spectra were made on a Hitachi U-3010 spectrophotometer (Tokyo, Japan). The pH was measured with a Model pHs-3C meter (Shanghai, China).

Reagents

The cationic cyanine dye was synthesized and purified according to the literature[24] and dissolved in methanol solution to make a 2.0 × 10^{-3} mol L^{-1} stock solution. The stock solution was then diluted to 2.0 × 10^{-5} mol L^{-1} with doubly distilled water as working solutions.

Fish sperm DNA (FS DNA) and calf thymus DNA (CT DNA) were purchased from the Sigma company. Stock solutions of nucleic acids were prepared by directly dissolving the commercial products in doubly distilled water. 24 h or more was needed and occasionally gently shaking the solution was made for complete dissolution at 0–4 °C. The concentration of all the working solutions for nucleic acids was 25 μg mL^{-1}.

All reagents were of analytical reagent grade without further purification. Water used throughout was doubly distilled water.

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Samples

Four synthetic samples were prepared based on the interference test of foreign substances.

Standard procedures

Into a 10 mL volumetric flask was transferred 0.5 mL of 2.0×10^{-5} mol L^{-1} cyanine working solution, 1.0 mL of hexamethylene tetramine-HCl buffer solution (pH 6.0), and an appropriate quantity of working solution or sample solution of nucleic acids was added. The mixture was diluted to 10 mL with water and thoroughly mixed. Then the fluorescence intensities of the sample (F) and the blank (F_0) solutions were measured with the following settings of the spectrofluorimeter: excitation wavelength, λ ex = 524.0 nm; emission wavelength, λ em = 591.5 nm; excitation and emission band-passes, 10 nm.

Results and discussion

Spectral characteristics

The hydrophobic cationic cyanine dye displayed a relatively weak fluorescence emission in aqueous solution with the excitation and emission peaks at 524 nm and 591.5 nm, respectively, compared with that in methanol. However, the fluorescence intensity was significantly enhanced with a bathochromic shift of maximal emission wavelength when CT DNA solution was added (Fig. 2).

The cyanine dye had a characteristic absorption peak at 524 nm in methanol, whereas the absorption maximum blue shifts to 508 nm in aqueous solution. In the presence of CT DNA, the absorbance scarcely changed but the absorption maximum red shifts from 508 nm to 532 nm with the increase of CT DNA concentration. The changes in absorption and fluorescence spectra of the cationic cyanine suggest the equilibrium shifts between the dye monomer and various aggregate forms, which resulted from some strong interaction between the cyanine dye and CT DNA. In terms of FS DNA, similar spectral characteristics could be observed.

Optimization of experimental conditions

All the factors which affect the sensitivity of the method and the stability of the system were investigated, including pH, concentration of the cyanine, salt effect and incubation time.

Effect of pH. The fluorescence enhancement of the system was greatly dependent on the pH since the pH had an effect on the nature of the cyanine and its binding ability to nucleic acids.[25] The effect of pH on the fluorescence intensity of the system in the presence (F) and in the absence of DNA (F_0) was studied in the range of pH 1.33 to 11.00 by employing NaAc–HCl, hexamethylene tetramine-HCl and H_3BO_3–NaOH media, respectively. The experiments indicated that the difference of fluorescence intensity in the absence and presence of DNA reached maximum and kept almost unchanged when the pH was in the range of 5.8–9.0 (Fig. 3A). The influence of different buffers *i.e.* Tris-EDTA and hexamethylene tetramine–HCl, on the sensitivity of this method was also compared and studied. The results showed that the sensitivity of the former was slightly high. In this work, we chose the pH 6.0 hexamethylene tetramine–HCl as the buffer system.

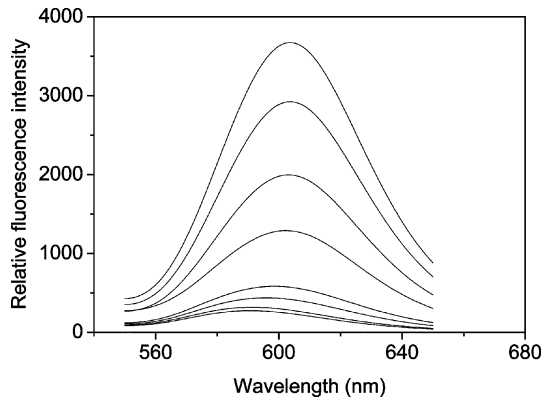

Fig. 2 The emission spectra of the cyanine in solutions with different concentrations of CT DNA. Concentration of CT DNA (From bottom to top): 0; 0.05; 0.5; 1.0; 3.0; 5.0; 8.0; 10.0 μg mL^{-1}. The excitation wavelength is 524 nm. Other conditions are the same as that described in the procedure.

Effect of the concentration of the cyanine. The experiments showed that the fluorescence intensity both in the absence and presence of DNA increased with increasing the concentration of the cyanine (Fig. 3B). However, the fluorescence difference in the absence and presence of DNA reached its maximum when the cyanine was at a concentration of 1.0×10^{-6} mol L^{-1} with a lower

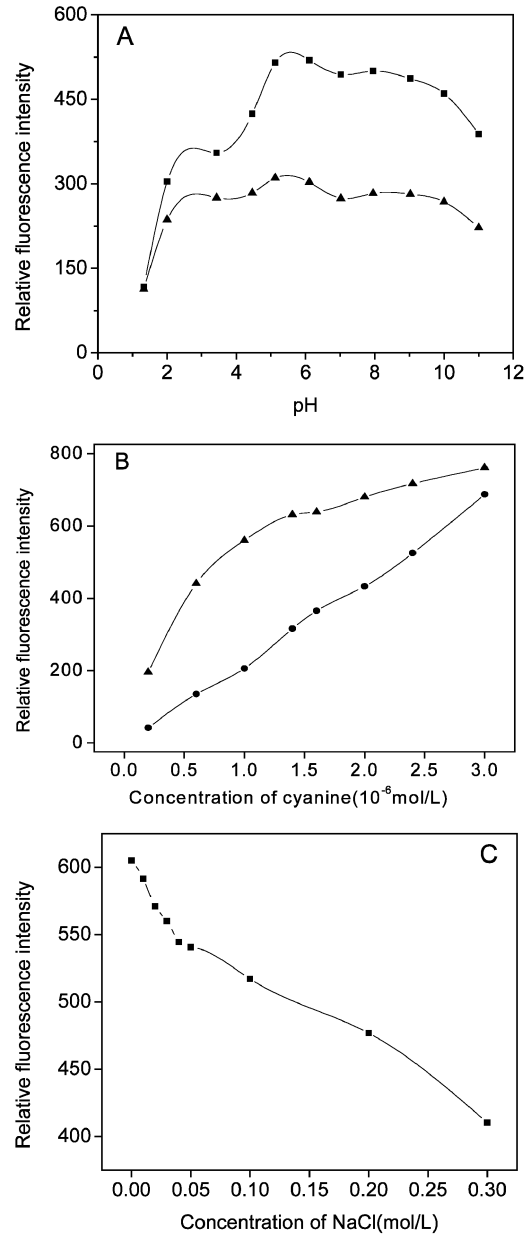

Fig. 3 (A) Effect of pH on fluorescence intensity of the cyanine in the presence of DNA (top) and in the absence of DNA (bottom). (B) Effect of cyanine concentration on fluorescence intensity of the cyanine in the presence of DNA (top) and in the absence of DNA (bottom). (C) Effect of ionic strength on fluorescence intensity of the cyanine in the presence of DNA. Concentration of CT DNA is 1.0 μg mL^{-1}. Other conditions are as described in the procedure.

background fluorescence. So 0.5 mL of the 2.0×10^{-5} mol L^{-1} working solution of cyanine was chosen for the assay.

The effect of ionic strength. The effect of salt on the assay was investigated by adding the strong electrolyte, sodium chloride. From Fig. 3C, it can be seen that with the increase in the concentration of NaCl, the fluorescence enhancement in the presence of DNA decreases rapidly, indicating that the interaction between cyanine and DNA is blocked. This result could be ascribed to the fact that ionic strength might result in the anion of phosphate on the backbone being shielded by the cation ion of the ionic strength controller (Na^+) when the ionic strength was too large.[26]

The incubation time. The incubation time was also studied. The results showed that the maximal fluorescence enhancement for different nucleic acid concentrations was all immediately reached when the solutions were mixed and the fluorescence remained almost constant for at least 2 h. In this work, the fluorescence intensity was measured within 30 min.

Tolerance of foreign substances

The influence of various ions, amino acids and proteins was studied (see Table 1). It can be seen that among the various ions and other substances tested, Cd(II), Pd(II) scarcely interfered, compared with other ions. Although other ions tested can be tolerated at a low level, their quantities in samples of biological fluids diluted for analysis are usually below the amounts tolerated under the experimental condition. It is also noted that EDTA can reduce the interference from some cations to some extent (see Table 1). Protein, the general main impurity in DNA samples, gives less interference on the method when their content is less than 10%. In addition, amino acids could be allowed at relatively high concentration levels. All the results showed that the present method had a satisfactory selectivity.

Table 1 Tolerance of foreign substances[a]

Foreign substances	Maximum concentration/ μg mL^{-1}	Relative error caused (%)
DL-aspartate	100	+5.0
Glysine	100	+1.7
L-leucine	100	+1.0
DL-α-aminopropionic acid	100	+2.1
L-valine	100	+0.5
L-serine	100	+0.1
L-proline	100	+3.1
L-lyrosine	100	+0.9
L-phenylalanine	50	+4.8
L-arginine	50	−3.4
L-cysteine	10	+4.6
$Cd^{2+}(NO_3^-)$	3	+1.5
$Pb^{2+}(NO_3^-)$	1.0	−0.7
$Mg^{2+}(Cl^-)$	0.1	+2.5
$Mg^{2+}(Cl^-)$ + EDTA	2	−4.5
$Zn^{2+}(Cl^-)$	0.1	−0.2
$Zn^{2+}(Cl^-)$ + EDTA	3	−1.3
$Ba^{2+}(Cl^-)$	0.5	+1.8
$Ba^{2+}(Cl^-)$ +EDTA	5	+3.1
$Ca^{2+}(Cl^-)$	0.1	+0.4
$Ca^{2+}(Cl^-)$ + EDTA	3	−4.7
$Mn^{2+}(Cl^-)$	0.1	+3.5
$Mn^{2+}(Cl^-)$ + EDTA	5	+1.2
$Fe^{3+}(Cl^-)$	0.5	−1.4
$Fe^{3+}(Cl^-)$ +EDTA	5	−4.6
$Al^{3+}(Cl^-)$	0.1	−1.3
$Al^{3+}(Cl^-)$ + EDTA	0.5	−5.0
BSA	0.1	−1.4
γ-IgG	0.1	−1.1
HSA	0.1	+3.3

[a] CT DNA: 1.0 μg mL^{-1}; EDTA: 1.0×10^{-4} mol L^{-1}.

Calibration curves and the determination of synthetic and real samples

The calibration graphs for the determination of nucleic acids were constructed under the optimal conditions (Fig. 4). All the analytical parameters were presented in Table 2.

The limit of detection (LOD) was given by the equation, LOD = KS_0/S, where K was a numerical factor chosen according to the confidence level desired, S_0 was the standard deviation of the blank measurements (n = 9) and S was the sensitivity of the calibration graph.[27] Here a value of 3 for K was used.

The relative standard deviation was 2.3% for determinations (n = 6) of DNA solution with a concentration of 1.0 μg mL^{-1} (3×10^{-6} mol L^{-1}) CT DNA. From Table 2 and Table 3, it was not difficult to see that the method had high sensitivity, a wide linear range and good reproducibility.

Compared with other methods for the determination of DNA, this method had the widest linear range and relatively lower limit of detection (see Table 3).

Four synthetic samples, prepared based on the interference test of foreign substances (Table 1), were analyzed according to the results in Table 2. The results were presented in Table 4.

Nucleic acids real samples, offered by the Department of Biology of Anhui Normal University, were diluted by 5000-fold

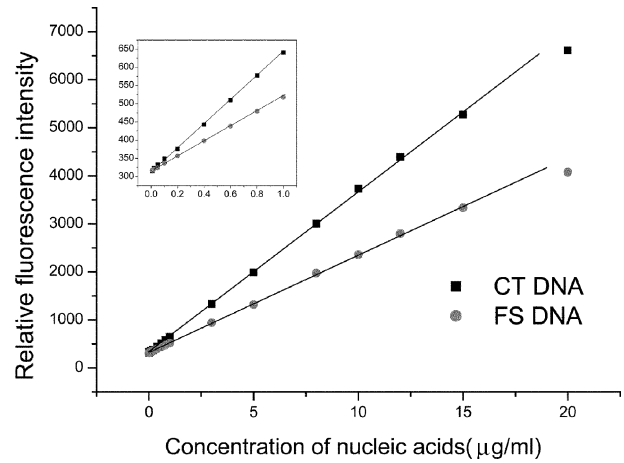

Fig. 4 Fluorescence intensity of the cyanine–DNA system *vs.* DNA concentration.

Table 2 Analytical parameters for the determination of nucleic acids

Nucleic acids	Linear range/ μg mL^{-1}	Linear regression equation (c)/μg mL^{-1}	Detection limit (3δ)/ μg mL^{-1}	Correlation Coefficient (r)
CT DNA	0.01–15	$\Delta F = 7.01 + 336.55c$	0.005	0.9998
FS DNA	0.01–15	$\Delta F = 6.39 + 203.88c$	0.007	0.9998

Table 3 Comparison of methods for determination of DNA

Method	$\lambda_{ex}/\lambda_{em}$/nm	LOD/ μg mL	Linear range/μg mL
Ethidium bromide[2]	546/590	0.01	—
Ethidium bromide[42]	520/590	0.033	0–12
Hoechst 33258[3]	356/492	0.01	0–15
Eu^{3+}-tetracycline[9]	398/615	0.01	0.02–1.0
Tb^{3+}-phenanthroline[8]	298/543	0.1	0.4–15
TOTO[13]	488/535	0.0005	0.0005–0.1
YOYO[13]	470/510	0.0005	0.0005–0.1
Cationic cyanine[23] [a]	765/790	0.03	0.1–1.2
Heptamethylene cyanine[27]	766/796	0.0068	0.01–0.25
This method	524/591.5	0.005	0.01–15

[a] [-4′-chloro-7′-(1″-ethyl-3″,3″-di-methyl-indolin-2″-ylidene)-3′,5′-(propane-1,3-diyl)-1′,3′,5′-heptatrien-1′-yl]-1-ethyl-3″ ′,3″ ′-dimethyl-3H-indolium iodide

with water just before determination without further pretreatment. Table 5 displayed the determination results for four nucleic acids real samples. The results of recovery tests whether for synthetic samples or real samples were satisfying. Therefore, the determination of nucleic acids by this method was reliable and practical.

Study on the interaction mechanism of the cyanine with nucleic acid

An important feature of cyanine dyes was their ability to aggregate. Cyanines are unique compounds which form aggregates of various compositions and structures in solutions, films and layers.[28–32] In many cases, cyanine dyes existed not as isolated monomers but rather as aggregates of multiple chromophores. Generally, cyanine dyes formed two types of aggregates, J- and H-aggregates, depending upon the geometry of aggregation. J- and H-aggregates resulted in staggered and stacked aggregation of the dye monomers, respectively.[28,33,34] J- and H-aggregates were well characterized by (1) the exhibition of intense red-shifted[31] and blue-shifted[35] absorption peaks; (2) strong[36] and weak[37] fluorescence; and (3) reduced and increased fluorescence lifetimes,[38] respectively, compared to monomers.

When hydrophobic cationic cyanine with great aggregation capability was used in solutions without DNA, it was expected that higher fluorescence enhancement in solutions with DNA could be obtained.[39] Such dyes displayed low inherent fluorescence in aqueous solution due to their strong aggregation ability, where the dyes mainly existed in aggregates, likely, H-aggregates. In aqueous solutions containing DNA, aggregates of dyes were dissociated, the dyes existed mainly in monomer, and thus showed strong fluorescence. Intense red-shifted absorption and dramatic fluorescence enhancement observed in our experiments directly supported the above viewpoint. It was also noted that cyanine dyes could spontaneously assemble into helical J-aggregates in the presence of a double-helical DNA template,[40,41] which may also cause the fluorescence enhancement and the intense red-shift of the absorption peak. Therefore, the spectral change of the hydrophobic cyanine in the presence of DNA could be attributed to the following possible reasons: (i) the cyanine was bound in the form of monomer into the minor groove of DNA, and the wall of the minor groove inhibited the excited-state twisting and the nonradiative decay of the dye; (ii) the cyanine dye spontaneously assembled into the double-helical DNA template to form helical J-aggregates. Apparently, further experiment is needed in order to reveal the mechanism of the interaction between the cyanine dye and nucleic acids. Nevertheless, it can be concluded that the interconversion between the dye monomer and various dye aggregates occurred and was facilitated when DNA was added into an aqueous solution of the cyanine. The ionic strength dependence shown in Fig. 3C suggested that the binding of free dye monomer with DNA and the formation of J-aggregates was severely inhibited, which resulted in the difficulty of the above interconversion, when the concentration of NaCl was too large.

Table 4 Determination results of synthetic samples

Samples	DNA concentration/ μg mL^{-1}	Foreign substances[a]	Found[b]/ μg mL^{-1}	Recovery (%, n = 6)
CT DNA	1.0	Cd^{2+}, L-leucine	1.08	105.2–110.5
CT DNA	1.0	Pb^{2+}, Cr^{3+}, Mg^{2+}	1.02	98.7–104.6
FS DNA	1.0	Cd^{2+}, L-serine	1.01	99.1–102.5
FS DNA	1.0	L-serine, Mg^{2+}, Zn^{2+}	1.02	97.6–105.8

[a] Cd^{2+}, Pb^{2+}, Cr^{3+}, Mg^{2+}, Zn^{2+}: 0.1 μg mL^{-1}; L-leucine, L-serine: 4.0 μg mL^{-1}. [b] Mean of six determinations.

Table 5 Determination results of real samples

Sample No.	Content of DNA[a]/ μg mL^{-1}	DNA added/ μg mL^{-1}	DNA found/ μg mL^{-1}	RSD (%) (n = 6)	Recovery of DNA (%) (n = 6)
1 chook DNA	0.70	1.0	1.73	2.1	103
2 duck DNA	0.36	1.0	1.41	1.9	105
3 crocodile DNA	0.25	1.0	1.21	2.8	96
4 crocodile DNA	0.53	1.0	1.54	1.3	101

[a] Mean of six determinations by this method.

Conclusions

A fluorescence enhancement method with a hydrophobic cationic cyanine as the probe was proposed for the determination of nucleic acids in aqueous solution. In addition to its stability, reproducibility and rapidity, this method had a wide linear range and low detection limit.

Acknowledgements

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