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Near-infrared fluorimetric determination of nucleic acids by shifting the ion-association equilibrium between tetracarboxy aluminum phthalocyanine and tetra-*N*-hexadecylpyridiniumyl porphyrin

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Abstract

A new method was developed for determination of micro amounts of nucleic acids based on near-infrared (near-IR) fluorescence recovery, employing a two-reagent system which is composed of an anionic tetracarboxy aluminum phthalocyanine (AlC_4Pc) and a cationic tetra-*N*-hexadecylpyridiniumyl porphyrin ($TC_{16}PyP$). The fluorescence of the AlC_4Pc, with the maximum emission wavelength at 701 nm, could be quenched by $TC_{16}PyP$ at its proper concentration, but recovered by adding nucleic acids. Under optimal conditions, the recovered fluorescence is proportional to the concentration of nucleic acids. The calibration graphs are linear over the range of 1–200 ng mL^{-1} for fish sperm DNA (FS DNA) and 2–400 ng mL^{-1} for calf thymus DNA (CT DNA). The corresponding detection limits are 0.59 ng mL^{-1} for FS DNA and 0.82 ng mL^{-1} for CT DNA, respectively. Four synthetic and three real nucleic acid samples were determined with satisfactory results.

Keywords: Near-IR fluorescence; Tetracarboxy aluminum phthalocyanine (AlC_4Pc); Tetra-*N*-hexadecylpyridiniumyl porphyrin ($TC_{16}PyP$); Nucleic acids

1. Introduction

Nucleic acids have an important function in life process, so research on them has become an important component of life science. Quantitative determination of nucleic acids is the base of studying nucleic acids. The natural fluorescence of nucleic acids is so weak that the direct use of their fluorescence emission properties has been limited. Therefore, extrinsic fluorescence probes must be employed. Several of the most widely used involve staining with a dye such as ethidium bromide [1]. Other dyes such as bisimidazole (Hoechst 33258) [2,3], 4,6-diamidino-2-phenylindole-HCl [4,5], yellow orange dyes [6], TOTO and YOYO [7], etc., also have been used as nucleic acids probes. However, ethidium bromide is a strong carcinogenic reagent, its intrinsic emission may cause significant interference when the concentration of nucleic acids is at a low level, and the other reagents mentioned all fluorescence in the visible region, a region of much interference by most bio-organic molecules, and yet they are too expensive for general uses in determination of nucleic acids.

In recent years, interest has focused on near-infrared (near-IR) probes (700–1200 nm), such as trivalent lanthanide [8,9] and some cyanine dyes [10,11], which are characteristic of low background interference, high molar absorbility, and high fluorescence quantum yield [12]. In our previous work, we synthesized a near-IR fluorescent probe, tetracarboxy aluminum phthalocyanine (AlC_4Pc, see Fig. 1, left), and investigated its potential possibility for the bio-macromolecule assay. Unfortunately, AlC_4Pc could not be directly employed to determine nucleic acids due to a lack of interaction between AlC_4Pc and DNA. In fact, a lot of dyes could not be directly used as nucleic acid probes since there are no or too weak interactions between the dyes and the nucleic acids to yield spectral shifts. In this case, it is a good strategy to choose and employ a proper association equilibrium system to determine nucleic acid

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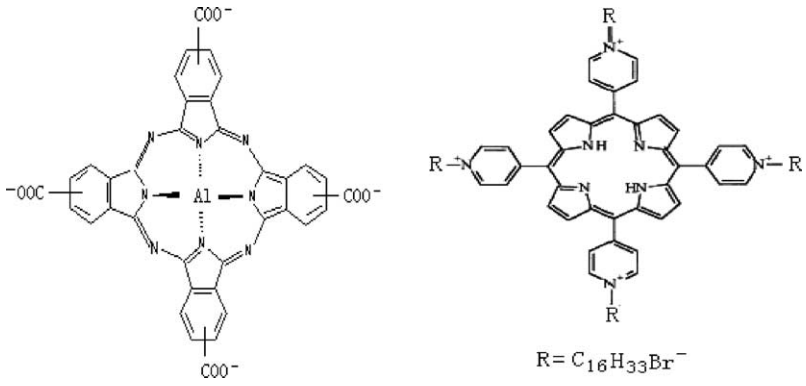

Fig. 1. Molecular structure of AlC_4Pc (left) and $TC_{16}PyP$ (right).

indirectly [13]. Accordingly we have ever reported a ternary association equilibrium system for the DNA analysis [14], in which, anionic tetrasulfonated aluminum phthalocyanine (AlC_4Pc) and cationic dye, Acridine Orange (AO) have been employed. The main advantage of this method is that the measurement is located at the near-IR range, a less spectral interference range.

Recently we synthesized a cationic porphyrin (tetra-*N*-hexadecylpyridiniumyl porphyrin, $TC_{16}PyP$, see Fig. 1, right), and investigated its interaction with DNA employing a resonance light scattering technique [15]. As a structural analogy of phthalocyanine, cationic $TC_{16}PyP$ is expected to more intensely interact with oppositely charged AlC_4Pc than other cationic dyes such as AO, and develop a more sensitive ternary association equilibrium system for the DNA analysis.

It supported our assumption that the anionic AlC_4Pc is highly fluorescent when free in solution. In the presence of positively charged $TC_{16}PyP$, the fluorescence of the AlC_4Pc is almost completely quenched. If nucleic acids are added, the AlC_4Pc regains its original fluorescence. Based on this phenomenon, a near-IR fluorescence recovery method for the determination of nucleic acids in aqueous solution has been developed. In addition to its simplicity, rapidity and stability, the method is highly sensitive. As revealed by the investigation below, this method has higher sensitivity compared with what we early reported [14,16] for the near-IR measurement of DNA employing ternary association equilibrium systems.

2. Experimental

2.1. Apparatus

A Hitachi F-4500 spectrofluometer (Tokyo, Japan) equipped with a R3896 red-sensitive multiplier 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).

2.2. Reagents

The anionic phthalocyanine dye was synthesized and purified according to the literature [17] and dissolved in 0.1 mol L^{-1} NaOH solution to yield a 2.0×10^{-3} mol L^{-1} stock solution which was then dilute to 2.0×10^{-5} mol L^{-1} with double distilled water as working solutions.

$TC_{16}PyP$ was synthesized and purified according to Ref. [18], and the stock solution was prepared by dissolving 0.1838 g of $TC_{16}PyP$ in about 15 mL dimethyl sulfoxide (DMSO) then diluting to 100 mL with doubly distilled water. The concentration of the stock solution was 1.0×10^{-3} mol L^{-1} and the working solution was obtained by further diluting.

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 double distilled water. Twenty-four hours or more and occasionally gently shaking were needed for complete dissolution at 0–4 °C. All working concentrations of nucleic acids were 10 μg mL^{-1}.

Hexamethylene tetramine–HCl buffer solution (pH 8.36, composed of 0.2 mol L^{-1} hexamethylene tetramine) was used to control the acidity.

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

2.3. Samples

Considering the possible interference from some foreign substances, four synthetic samples were constructed, through mixing standard DNA solutions (50 ng mL^{-1}) with various foreign substances such as metal ions, amino acids and proteins.

2.4. Standard procedures

To a 10 mL volumetric flask 0.2 mL of hexamethylene tetramine–HCl buffer solution (pH 8.36), 0.1 mL 1.0×10^{-4} mol L^{-1} $TC_{16}PyP$ solution, and an appropriate

working solution (10 μg mL^{-1}) or sample solution of nucleic acids were transferred, then 0.1 mL of 2.0 × 10^{-5} mol L^{-1} phthalocyanine working solution 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) (prepared in a similar manner without DNA) solutions were measured with the following settings of the spectrofluorimeter: excitation wavelength, λ_{ex} = 620.0 nm; emission wavelength, λ_{em} = 701 nm; excitation slit, 5 nm; emission slit, 10 nm; photomultiplier voltage, 700 V.

3. Results and discussion

3.1. Spectral characteristics

AlC_4Pc displayed an intense fluorescence in aqueous solution with an emission maximum at 701 nm when the excitation wavelength was set at 620 nm (Fig. 2, curve 1). The fluorescence was significantly quenched in the presence of $TC_{16}PyP$ (Fig. 2, curve 2). When nucleic acids were added to the AlC_4Pc–$TC_{16}PyP$ system, the fluorescence intensity of the system gradually recovered without obvious wavelength change (Fig. 2, curves 3–5).

AlC_4Pc displayed an intense absorption in aqueous solution with a maximum absorption at 688 nm (Fig. 3, curve 1). When different concentrations of $TC_{16}PyP$ were mixed with AlC_4Pc, no significant wavelength shift occurred, but the absorption intensity decreased with increasing amounts of $TC_{16}PyP$ (Fig. 3, curves 2–5), indicating a strong interaction occurred between them.

Under the experimental conditions, $TC_{16}PyP$ is cationic, whereas AlC_4Pc is anionic. Therefore the electrostatic interaction resulted in the formation of anionic–cationic association, a large aggregate, reducing the amount of the free form of AlC_4Pc, which accounts for the absorption decrease and fluorescence quenching of AlC_4Pc.

As shown in Fig. 4, the absorbance of AlC_4Pc increased with increasing amounts of FS DNA, however, the absorption maximum at 688 nm remains unchanged, suggesting that the ion-association equilibrium between AlC_4Pc and $TC_{16}PyP$ was destroyed and accordingly the amount of free form of AlC_4Pc increased due to the presence of DNA.

Considering that DNA scarcely changed the absorption or fluorescence spectra of AlC_4Pc (spectra not shown), the explanation for above spectral change is the shifting of the

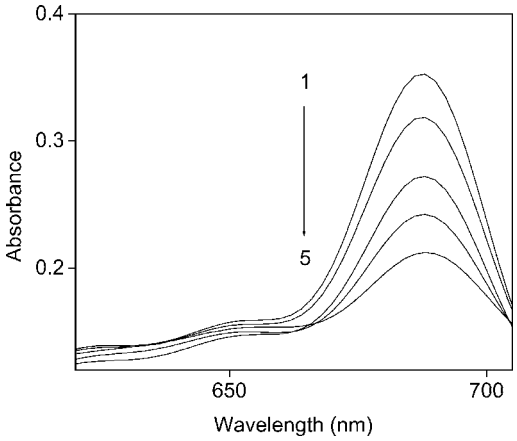

Fig. 3. Absorbance spectra of AlC_4Pc in the absence (curve 1) and presence of $TC_{16}PyP$ (curves 2–5). The concentration of $TC_{16}PyP$ (from curves 2 to 5): 0.2; 0.35; 0.5; 0.75 × 10^{-6} mol L^{-1}. Other conditions are the same as that described in the procedure.

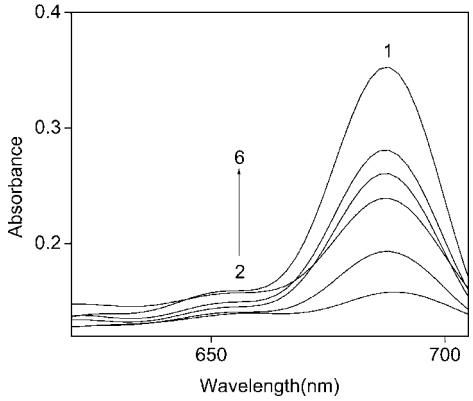

Fig. 4. Absorption spectra of AlC_4Pc (curve 1) and the AlC_4Pc–$TC_{16}PyP$ system with different concentration of FS DNA (curves 2–6). The concentration of FS DNA (from curves 2 to 6): 0; 50; 100; 200; 500 ng mL^{-1}. Other conditions are the same as that described in the procedure.

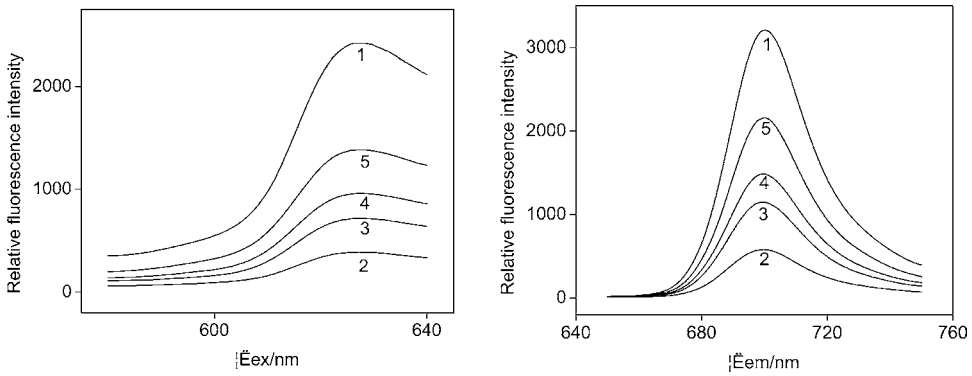

Fig. 2. The excitation (left) and emission (right) spectra of AlC_4Pc (curve 1) and the AlC_4Pc–$TC_{16}PyP$ system with different concentration of FS DNA (curves 2–5). Concentration of FS DNA (from curves 2 to 5): 0; 50; 100; 200 ng mL^{-1}. Other conditions are the same as that described in the procedure.

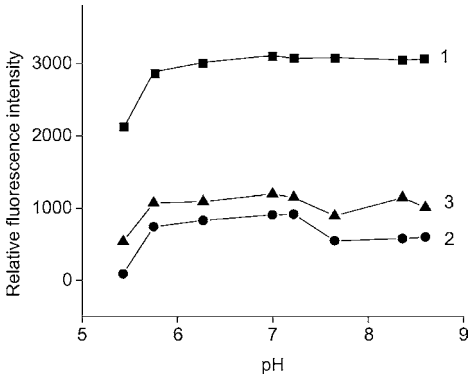

Fig. 5. Effect of pH. 1. AlC_4Pc; 2. $AlC_4Pc + TC_{16}PyP$; 3. $AlC_4Pc + TC_{16}PyP + FS$ DNA. The concentration of FS DNA is 50 ng mL^{-1}. Other conditions are the same as that described in the procedure.

ion-association equilibrium between AlC_4Pc and $TC_{16}PyP$ in the presence of DNA.

3.2. Buffer system and pH

In terms of the analytical sensitivity and linear range of the calibration graph, it was found that hexamethylene tetramine–HCl buffer was better than phosphate buffer. In the latter, the amounts of PO_4^{3-} ions (negatively charged) might inhibit the interaction of $TC_{16}PyP$ with nucleic acids, which made it difficult to replace AlC_4Pc from $TC_{16}PyP$ for low concentration DNA.

An investigation was also performed into the influence of pH on the sensitivity of the method since pH had an effect on the charge of AlC_4Pc, $TC_{16}PyP$ and DNA to some extent. The best sensitivity was obtained with hexamethylene tetramine–HCl buffer of pH 8.36 (see Fig. 5). In addition, the amounts of the buffer did not show any obvious effect on the value of $F–F_0$ when the concentration of hexamethylene tetramine is below 50 mmol L^{-1}. So 0.2 mL hexamethylene tetramine solution was chosen for the assay.

3.3. Effect of the order of addition of reagents

An investigation was carried out in order to ascertain the best order for adding the reagents. Experiments showed that the order of buffer, $TC_{16}PyP$, DNA, and AlC_4Pc was a suitable one.

3.4. Usage of AlC_4Pc and $TC_{16}PyP$

It is expected that too low concentration of AlC_4Pc and $TC_{16}PyP$ would lead to a limit fluorescence recovery since there are only a small amounts of AlC_4Pc completely replaced by DNA from $TC_{16}PyP$ matrix and existed as dye monomer, which is unfavorable to enlarging linear range. On the other hand, too high concentration of AlC_4Pc and $TC_{16}PyP$ would cause a low sensitivity, which may be ascribed to the difficulty to replace the AlC_4Pc from $TC_{16}PyP$ matrix for low concentration of DNA. In addition, it is also important to control the ratio of AlC_4Pc to $TC_{16}PyP$ concentration. A high ratio would cause a high background signal and a low sensitivity of the method since only a small percentage of phthalocyanine is in the aggregated form in this case, which results in a poor initial fluorescence quenching and thus a high background signal and low sensitivity. On the other hand, although low ratio of AlC_4Pc to $TC_{16}PyP$ permits us to start the measurement with extremely low background fluorescence intensity, low ratio would increase the probability of direct interaction of DNA with the $TC_{16}PyP$ matrix due to the number of binding sites available on the matrix. As a result, the system can not give a ready response to the first few DNA aliquots added until all binding sites on the matrix are saturated. In addition, if the ratio is too low, it may cause a narrow linear range. Thus, this effect causes a difficulty in the quantitative determination of DNA. Experiments showed that the best sensitivity would be achieved when the final concentrations of AlC_4Pc and $TC_{16}PyP$ were set at 2.0×10^{-7} and 1.0×10^{-6} mol L^{-1}, respectively.

3.5. Influence of incubation time

The influence of incubation time on the fluorescence recovery of the system was also tested. Results showed that the system reached equilibrium immediately and remained constant for at least 1 h. Therefore, a 5 min incubation time was recommended.

3.6. Effect of ionic strength

The effect of salt on the assay was investigated by adding the strong electrolyte, sodium chloride (NaCl). Results in-

Table 1
Tolerance of foreign substances

Foreign substances	Added (ng mL^{-1})	Concentration of FS DNA (ng mL^{-1})	Relative error (%)
$Ca^{2+}(Cl^-)$	300	50	−1.2
$Ba^{2+}(Cl^-)$	300	50	−1.6
$Cd^{2+}(NO_3^-)$	300	50	−2.1
$Mn^{2+}(Cl^-)$	300	50	−2.9
$Mg^{2+}(Cl^-)$	100	50	−3.2
$Zn^{2+}(Cl^-)$	100	50	−3.7
$Co^{2+}(Cl^-)$	50	50	−4.5
$Fe^{3+}(Cl^-)$	50	50	−2.4
$Ag^{+}(NO_3^-)$	50	50	−4.3
L-Arginine	6000	50	+0.9
DL-Aspartate	6000	50	+1.3
L-Proline	6000	50	+1.8
Glycine	6000	50	+3.2
L-Valine	3000	50	+3.0
DL-α-Aminopropionic acid	2000	50	+3.3
γ-IgG	100	50	+4.0
BSA	100	50	+4.6
HAS	50	50	+3.1
CTAB	200	50	+2.1
SDBS	200	50	+4.8
Triton X	200	50	−5.0

Table 2
Analytical parameters for the determination of nucleic acids

Nucleic acids	Linear range ($ng\,mL^{-1}$)	Linear regression equation (c, $ng\,mL^{-1}$)	Detection limit (3δ, $ng\,mL^{-1}$)	Correlation coefficient (r)
CT DNA	2–400	$\Delta F = 181.59 + 4.21c$	0.82	0.995
FS DNA	1–200	$\Delta F = 361.76 + 5.89c$	0.59	0.993

Table 3
Determination results of synthetic samples

Samples	DNA concentration ($ng\,mL^{-1}$)	Foreign substances	Found[a] ($ng\,mL^{-1}$)	Recovery (%, $n = 6$)
CT DNA	50	Glycine, Ba^{2+}, Mn^{2+}	49	95–102
CT DNA	50	L-Arginine, BSA, Ca^{2+}	48	94–99
FS DNA	50	Ca^{2+}, HSA	52	101–105
FS DNA	50	γ-IgG, HAS, Ba^{2+}	51	99–103

Glycine, L-arginine: $4000\,ng\,mL^{-1}$; Mn^{2+}, Ba^{2+}, Ca^{2+}: $300\,ng\,mL^{-1}$; γ-IgG, HSA, BSA: $50\,ng\,mL^{-1}$.
[a] Mean of six determinations.

Table 4
Determination results of real nucleic acid samples

Sample no.	Content of DNA[a] ($ng\,mL^{-1}$)	DNA added ($ng\,mL^{-1}$)	DNA found ($ng\,mL^{-1}$)	R.S.D. (%, $n = 6$)	Recovery of DNA (%, $n = 6$)
1. Crocodile DNA	48	50	96	2.9	96
2. Chook DNA	124	50	175	1.6	102
3. Duck DNA	72	50	121	2.4	98

[a] Mean of six determinations by this method.

dicated that NaCl had a strong effect on the fluorescence of sample solutions, because both phthalocyanine and DNA binding to $TC_{16}PyP$ is driven mainly by electrostatic interactions. Our experiments showed that the final concentration of salt in system should not higher than $50\,mmol\,L^{-1}$.

3.7. Interference of foreign substances

The effects of various foreign substances on the determination of $50\,ng\,mL^{-1}$ FS DNA by the procedure described were studied. For a relative error of less than ±5%, the tolerance of foreign substances is listed in Table 1. From Table 1 it can be seen that most of the tested substances scarcely interfered with the determination. Co(II), Fe(III) and Ag(I) can be tolerated at somewhat low levels, but their content in biological fluids or real samples is far below the tolerance listed when the sample is diluted for determination.

3.8. Calibration graphs and analysis of samples

The calibration graphs for two kinds of nucleic acids were constructed by performing the standard procedure under the optimum conditions and the results are given in Table 2.

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

The relative standard deviation ($n = 6$) was 2.1% for $50\,ng\,mL^{-1}$ CT DNA, 1.8% for $50\,ng\,mL^{-1}$ FS DNA, respectively. From Tables 1 and 2, it is not difficult to find the method is characteristic of high sensitivity and good reproducibility.

Four synthetic samples, prepared based on the interference test of foreign substances (Table 1), were analyzed according to the results in Table 2. The determination results were presented in Table 3.

Nucleic acids real samples, offered by the department of biology of Anhui Normal University, were diluted 25,000-fold with deionzed water just before determination without other pretreatment. Table 4 displays the determi-

Table 5
Comparison of methods for determination of DNA

Method	$\lambda_{ex}/\lambda_{em}$ (nm)	LOD ($ng\,mL^{-1}$)	Linear range ($ng\,mL^{-1}$)
Ethidium bromide [1]	546/590	10	–
Hoechst 33258 [2]	356/492	10	0–15000
TOTO [7]	488/535	0.5	0.5–100
YOYO [7]	470/510	0.5	0.5–100
Tb^{3+}-phenanthroline [8]	298/543	100	400–15000
Eu^{3+}-tetracycline [9]	398/615	10	20–1000
Magdala red [19]	540/555	7	10–1200
Brilliant cresyl blue [20]	626/670	7	20–800
Tetrasulfonated aluminum phthalocyanine [14]	615/688	24	10–1200
Heptamethylene cyanine [16]	766/796	6.8	10–250
This method	620/701	0.59	1–200

nation results for three nucleic acids real samples. From Tables 3 and 4 it can be seen that the results of recovery whether for synthetic samples or real samples were satisfying, suggesting that the method is reliable and practical.

4. Conclusions

A new method based on near-infrared fluorescence recovery, employing a two-reagent system which is composed of an anionic tetracarboxy aluminum phthalocyanine (AlC_4Pc) and a cationic tetra-*N*-hexadecylpyridiniumyl porphyrin ($TC_{16}PyP$), is presented for the determination of nucleic acids. In addition to its simplicity, rapidity and stability, the main advantage of this method is that the fluorescence is measured by means of a conventional fluorescence spectrophotometer in near-infrared region, a region of less interference from most biomolecules and matrix. Furthermore, from Table 5 it can be seen that, although the sensitivity of the method is a little lower than TOTO and YOYO, it shows higher sensitivity than other methods. Especially, this method has a higher sensitivity compared with what we early reported [14,16] for the near-IR measurement of DNA employing ternary association equilibrium systems.

Acknowledgements

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