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Application of magdala red as a fluorescence probe in the determination of nucleic acids

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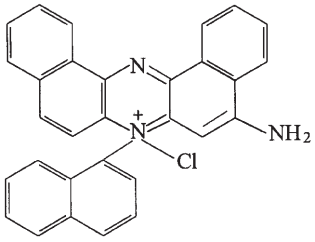
Abstract A fluorescence quenching method was developed for the rapid determination of DNA and RNA using magdala red as fluorescence probe. In weakly acidic medium, the fluorescence of magdala red (λex\λem = 540\555 nm) can be largely quenched by DNA or RNA. The calibration graphs are linear over the range 0.01–1.2 μg/mL for both calf thymus DNA (CT DNA) and salmon DNA (SM DNA), and 0.015–1.0 μg/mL for yeast RNA, respectively. The corresponding detection limits are 6.0 ng/mL for CT DNA, 7.0 ng/mL for SM DNA and 15.0 ng/mL for yeast RNA, respectively. CT DNA could be determined in the presence of 20% (w/w) yeast RNA, and the relative standard deviation of six replicate measurements is 3.18% for 400 ng/mL of CT DNA. Interference from coexisting substances in the determination of DNA was also examined. Real samples were determined with satisfactory results.

1 Introduction

In the quantitative analysis of nucleic acids, the fluorescence quantum yield of native DNA is so low [1] that it cannot be used for quantitative determination. Up to now, various fluorescence methods for the determination of nucleic acids have been established based on extrinsic fluorescent probes [2–5]. Several of the most widely used involve staining with a dye such as ethidium bromide [2]; however, the main limitation of ethidium bromide is its toxicity. In recent years, some new fluorescence probes, such as trivalent lanthanide cations [6–10] and dimeric asymmetric cyanine dyes [11, 12], have received much attention because of their long wavelength emission and high sensitivity; but these probes are expensive. Therefore, it is considered important to develop a sensitive and rapid method for the determination of DNA using an inexpensive probe.

Fig. 1 Structure of magdala red



Magdala red is a very common dye and displays strong fluorescence emission at 555 nm with maximum excitation at 540 nm. The structure of magdala red is shown in Fig. 1. In our research, it was found that its fluorescence was significantly quenched by DNA or RNA in acidic medium, and the fluorescence quenching showed a good linear relationship with the concentration of DNA or RNA. We have therefore employed magdala red as a fluorescence probe and developed a sensitive fluorescence quenching method for the determination of nucleic acids. The use of magdala red leads to a particularly inexpensive, simple and sensitive system, permitting a limit of detection of 6.0 ng/mL for CT DNA, 7.0 ng/mL for SM DNA and 15.0 ng/mL for yeast RNA, respectively.

2 Experimental

2.1 Apparatus. A Hitachi 650–10S spectrofluorimeter equipped with a plotter unit and a 1 cm quartz cell was used for recording fluorescence spectra and making fluorescence measurements. All pH measurements were made with a Model PHS-301 pH meter 631 (Xiamen, China).

2.2 Reagents. All chemicals were of analytical reagent grade or higher purity. All aqueous solutions were made up in distilled, deionized water.

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Commercially prepared calf thymus DNA, salmon DNA and yeast RNA, obtained from Sino-American Biotechnology Co. (Shanghai, China), were directly dissolved in water at a final concentration of 200 μg/mL and stored at 4 °C. These solutions were diluted to 20 μg/mL with water as working solutions. A magdala red stock solution (1.0×10^{-3} mol/L) was prepared by dissolving the appropriate weight of magdala red (Beijing Chemical Reagent Co., Beijing, China) into 100 mL of water and stored in the dark. This solution was diluted to 1.0×10^{-5} mol/L with water as working solution. A pH 4.0 HAc-NaAc buffer solution was prepared by mixing 18 mL of 0.2 mol/L NaAc and 82 mL of 0.2 mol/L HAc.

2.3 Procedure. 2.0 mL of buffer solution (pH 4.0) and 0.1 mL of magdala red solution (1.0×10^{-5} mol/L) were transferred to a 10 mL standard flask. A known volume of DNA (or RNA) standard solution was added. Then, the mixture was diluted to 10 mL with water and mixed. The relative fluorescence intensities of the reagent blank (F_0) and the mixed solution (F) were measured at 555 nm with excitation at 540 nm.

2.4 Treatment of real samples. The treatment of real samples was similar to the method reported previously [13]. In order to obtain DNA and RNA from the samples completely, a modified procedure was used. 0.1 g of dry pollen (or 0.3 g of honey) was mixed with 1 mL of 5% perchloric acid solution in a mortar box. The mixture was ground into homogenate at 0 °C, and centrifuged at 0 °C for 10 min (3000 rpm); then the precipitate was collected and washed with 1 mL of 5% perchloric acid at 0 °C for 10 min, followed by centrifugation. This procedure was repeated three times in order to remove acid-soluble proteins. 5 mL of ethyl alcohol/ethyl ether/chloroform mixing solvent (2:2:1, v/v) was added to the precipitate, the mixture was allowed to stand for 15 min at room temperature to extract phosphatide, then centrifuged (3000 rpm) to remove the extraction solution. The procedure was repeated four times, and the final precipitate of nucleic acids was dried in vacuo.

3.0 mL of 1.0 mol/L NaOH solution was added to the precipitate to hydrolyze nucleic acids at 25 °C for 8 h. The mixture was centrifuged and the centrifugate was washed with 1.0 mL of 1.0 mol/L NaOH twice, followed by centrifugation to remove the residue. All the centrifugates were mixed and acidified by adding 1.0 mL of 6.0 mol/L HCl solution, and then left at 4 °C overnight to precipitate DNA from the solution. After DNA was separated by centrifugation, the centrifugate, RNA moiety, was quantitatively transferred to a 25 mL standard flask and diluted to the mark with water. Then, 5.0 mL of 1.0 mol/L perchloric acid solution was added to the DNA precipitate to hydrolyze DNA at 75 °C for 20 min. The hydrolysate was centrifuged and the centrifugate (DNA moiety) was transferred to a 25 mL standard flask and diluted to the mark with water.

The DNA and RNA levels in the real samples were determined according to the procedure described above.

3 Results and discussion

3.1 Spectral characteristics

Magdala red is a strongly fluorescing dye. In a weak acidic medium, it displays an emission band located at 555 nm and an excitation peak at 540 nm, respectively. However, it was found that the fluorescence intensity of magdala red is significantly quenched in the presence of DNA (or RNA) (see Fig. 2). In contrast, the excitation and emission maxima of magdala red remain unchanged at 540 and 555 nm, respectively, even in the presence of an excess of CT DNA. RNA also exhibits a similar quenching effect on the fluorescence of magdala red; however, its quenching ability is lower than that of DNA. These results indicated that magdala red could be used as a new fluorescence probe for the sensitive determination of DNA. In this work, the maximum excitation peak at 540 nm and the emission peak at 555 nm were used for fluorescence intensity measurements.

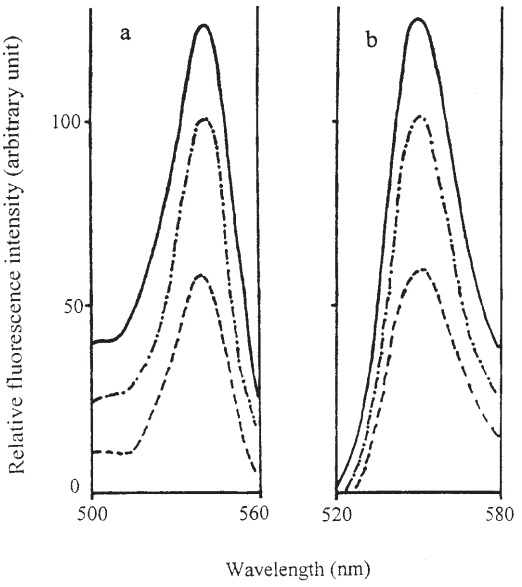

Fig. 2 Excitation (*a*) and emission (*b*) spectra of free magdala red (solid curves) and in the presence of yeast RNA (dot-dashed curves) and CT DNA (dashed curves). magdala red: 1.0×10^{-7} mol/L; CT DNA: 400 ng/mL; RNA: 400 ng/mL

3.2 Optimization of the general procedure

The experimental results indicated that maximum and constant fluorescence quenching was produced when the magdala red concentration was in the range 5.0×10^{-8} mol/L–2.0×10^{-7} mol/L. In this work, a magdala red concentration of 1.0×10^{-7} mol/L was used. The effect of pH on the fluorescence quenching of the system was studied. The fluorescence quenching reached a maximum over the pH range 3.8–4.2. Therefore, a pH of 4.0 was chosen for use, by addition of 2.0 mL buffer solution in 10 mL of final solution. The influence of incubation time on fluorescence quenching was also investigated. The results showed that the maximal fluorescence quenching was immediately reached when the solutions were mixed and remained constant for at least 2 h. In this work, the fluorescence intensity was measured directly after the solutions were mixed, and an additional incubation time was not needed. For RNA, the optimum conditions were similar to that of the DNA system.

3.3 Interference of foreign substances

The effects of various foreign substances on the determination of 400 ng/mL CT DNA by the procedure described were studied. For a relative error of less than ± 5%, the

Table 1 Tolerance of foreign substances in the determination of 400 ng/mL CT DNA

Coexisting substances	Coexisting conc. ($\times 10^{-6}$ M)	Relative error (%)	Coexisting substances	Coexisting conc. ($\times 10^{-6}$ M)	Relative error (%)
Thymine	3.18	+4.0	Al^{3+}(chloride)	0.10	–5.0
Adenine	0.74	+4.0	Zn^{2+}(chloride)	0.20	–3.6
Cytosine	3.10	+5.0	Ca^{2+}(chloride)	0.20	–3.4
Guanine	2.60	+2.2	Mg^{2+}(chloride)	1.00	–4.9
BSA	0.25	+3.7	Fe^{3+}(sulfate)	0.10	–5.0
HAS	0.13	+4.2	Co^{2+}(sulfate)	0.06	–5.1
IgG	3.00	+4.3	Cu^{2+}(sulfate)	0.10	–5.3
EDTA	4.00	–5.0	Ba^{2+}(chloride)	0.02	–4.3
SDS	2.00	–4.4	Pb^{2+}(nitrate)	0.04	–4.0

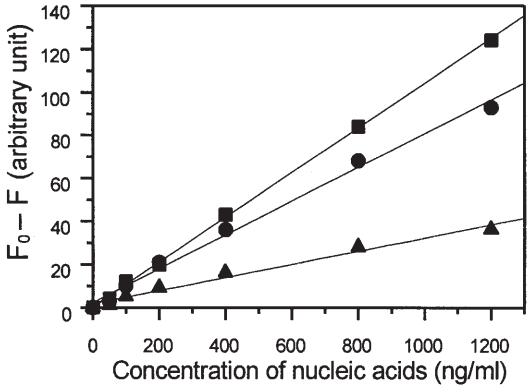

Fig. 3 Calibration curves for CT DNA (■), SM DNA (●) and yeast RNA (▲). For conditions, see procedure

tolerance of foreign substances is listed in Table 1. It can be seen that the tolerance of coexisting purine or pyrimidine bases is relatively high, i.e., these bases may not interfere with the determination of DNA in the proposed method. However, some foreign ions, such as Pb^{2+}, Ba^{2+}, seem to show low tolerance levels. Therefor, a separation procedure is needed before the determination of nucleic acids in complex biological samples in order to eliminate the interference.

3.4 Calibration graphs

The calibration graphs for the determination of DNA and RNA were constructed under the optimized conditions. The results are shown in Fig. 3. It can be seen that the extents of fluorescence quenching show good linear relationships to the DNA (or RNA) concentrations. All the analytical parameters are presented in Table 2. It clearly shows that the sensitivity for the determination of CT DNA is higher than that for SM DNA and yeast RNA with the sequence CT DNA > SM DNA > yeast RNA.

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.

Table 2 Analytical parameters for the determination of nucleic acids

Nucleic acid	Linear range (ng/mL)	Linear regression equation (C: ng/mL)	LOD (ng/mL)	r
CT DNA	0–1200	F = 0.10 C + 0.21	6.0	0.9987
SM DNA	0–1200	F = 0.09 C + 1.00	7.0	0.9974
Yeast RNA	0–1000	F = 0.03 C + 0.45	15.0	0.9985

Table 3 Comparison of methods for the determination of DNA

Method	Incubation time (min)	LOD (ng/mL)	Linear range (μg/mL)	RSD (%)
Ethidium bromide	–	10	–	–
Hoechst 33258	–	10	0–15	–
Tb^{3+}-phenanthroline	20–30	100	0.4–15	3.0
Eu^{3+}-tetracycline	–	10	0.02–1.0	3.0
TOTO	20–30	0.5	0.0005–0.1	–
YOYO	20–30	0.5	0.0005–0.1	–
This method	no[a]	6.0	0–1.2	2.0

[a] The maximal fluorescence quenching was immediately reached when the solutions were mixed, the additional incubation time was not needed

3.5 Comparison of the methods

Some characteristics of the proposed method and other methods for nucleic acid determinations are summarized in Table 3. It can be seen that the sensitivity of the proposed method is higher than that of classical dyes such as ethidium, Hoechst 33258 and lanthanide cations. However, the cyanine dyes, such as TOTO, YOYO, etc., are more sensitive compared to magdala red, but they show narrow linear range and a longer incubation time.

3.6 Determination of nucleic acids in synthetic samples

As described above, DNA has a much greater ability to quench the fluorescence of magdala red. So, it is expected that DNA could be measured in the presence of RNA. The determinations of CT DNA in synthetic samples, which contained various concentrations of RNA, were carried out according to the experimental procedure. The results

Table 4 Determination of CT DNA in synthetic samples

Sample no.	Composition of samples (ng/mL)	Recovery of DNA[a] (%)	RSD (%)
1	DNA (200)	101.5	3.93
2	DNA (400)	102.8	3.18
3	DNA (400) + RNA (20)	103.1	2.28
4	DNA (400) + RNA (40)	104.1	2.42
5	DNA (400) + RNA (80)	105.5	2.39
6	DNA (400) + RNA (160)	124.4	1.93

[a] Average of six determinations

Table 5 Determination of yeast RNA in synthetic samples

Sample no.	Composition of samples (ng/mL)	Recovery of RNA[a] (%)	RSD (%)
1	RNA (100)	104.7	1.84
2	RNA (200)	96.5	2.06
3	RNA (200) + DNA (10)	128.6	1.21
4	RNA (200) + DNA (20)	166.2	1.47

[a] Average of six determinations

are shown in Table 4. It can be observed that CT DNA could be determined in the presence of up to 20% RNA (w/w) with satisfactory results.

The above procedure was also applied to the determination of RNA in four synthetic samples (Table 5). It was found that the presence of CT DNA seriously interfered with the determination of RNA even when the concentration ratio of CT DNA to RNA in the samples was 5%.

3.7 Application to DNA and RNA determination in real samples

The DNA and RNA levels in pollen and honey samples were determined by the proposed method. Although the proposed method showed a certain selectivity, large quantities of coexisting substances in real samples, such as proteins, ions etc., may interfere with the determination of nucleic acids, in addition to which DNA interferes with the determination of RNA too. It is therefore necessary to separate DNA and RNA from the interfering substances and prepare DNA and RNA extraction solutions, respectively. Generally, since DNA and RNA exist in the cell nucleus, a separation and extraction procedure for nucleic acids must be involved prior to their determination with spectrophotometry and/or fluorimetry. In the proposed method, the extraction of DNA and RNA from the real samples was carried out by a procedure similar to that reported by Zhang [13], which showed high efficiency in the extraction of nucleic acids. The analytical results obtained by the proposed method are summarized in Table 6. In order to prove the possibility of using this method for the analysis of real samples, the same extraction solutions were also analyzed by two conventional spectrophotometric methods [13, 14] (Table 6). It can be seen that the values obtained by the different methods are in good agreement.

Table 6 Determination of DNA and RNA in real samples

Sample No.	DNA levels (mg/g)[a]		RNA levels (mg/g)[a]	
	This method	Spectrophotometry[b]	This method	Spectrophotometry[c]
1	0.42	0.37	7.40	7.80
2	0.49	0.48	10.0	11.80
3	0.08	0.07	1.74	2.04
4	36.4	34.0	7.40	6.70

[a] Average of six determinations
[b] Ref [14], use diphenylamine as a chromogenic reagent, DNA was determined at 595 nm
[c] Ref [13], RNA was directly determined at 260 nm

4 Conclusions

The proposed fluorescence quenching method demonstrates high sensitivity for the determination of nucleic acids with magdala red as a new fluorescence probe. In acidic medium, the fluorescence of magdala red is largely quenched by nucleic acids. In addition to its sensitivity, other advantages of this method are simplicity, stability and rapidity. The possibility of using this method for the analysis of DNA (or RNA) was tested using synthetic samples and real samples with satisfactory results.

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