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Fluorescence enhancement of terbium(III) by nucleotides and polyhomonucleotides in the presence of phenanthroline

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Summary. The fluorescence enhancement of terbium(III) by nucleotides (AMP, ADP, ATP, GMP, GDP, GTP) and polyhomonucleotides [poly(A), poly(G), poly(C), poly(U)] in the presence of phenanthroline (phen) was studied. Investigation of the composition of the terbium(III)/ANP(AMP, ADP, ATP)/phen complexes and conditions of optimization suggest a 1:2 molar ratio of terbium(III) and phen for the ternary complexes. The results showed that the presence of phen enhanced the net fluorescence of terbium(III)/ANP, poly(A), poly(C) or poly(U) from several fold to more than one-hundred fold, while it has little effect on the fluorescence of terbium(III)/GNP(GMP, GDP and GTP) or the poly(G) system. The possibility of spectrofluorimetric measurements of these compounds were studied under optimal conditions (pH 7.0 in tris-HCl buffer; $E_x = 298$ nm, $E_m = 543.5$ nm). The detection limits were 2.0×10^{-7}, 6.0×10^{-7} and 1.0×10^{-6} mol/l for AMP, ADP and ATP, respectively. The relative standard deviations (6 replicates) were within 2.0% in the middle of the linear range.

Introduction

Luminescent trivalent lanthanide ions have recently found increasing use as probes of nucleic acids and nucleotides [1–5]. In particular, attention has been directed towards two rare earth cations, terbium(III) and europium(III), as their resonance energy levels overlap with the triplet energy states of nucleic acid ligands irradiated with ultraviolet light [6]. The investigations previously reported revealed that, among the common five bases, guanine, in both mono- and polynucleotide forms, was found to enhance the terbium(III) fluorescence far more effectively than the ribo- or deoxyribonucleotides of the other four bases, and, therefore, the fluorescence enhancement of terbium(III) is mainly due to the guanine residue in nucleic acids. During our investigation on the fluorescence enhancement of terbium(III) by nucleic acids we found that in the presence of phen the net fluorescence of terbium(III) was enhanced 3–12 fold by thermally denatured nucleic acids (calf thymus DNA, fish sperm DNA and yeast RNA). To understand this phenomenon in the nucleotides level it is reasonable to study the common bases separately. This paper deals with the study of six nucleotides and four polyhomonucleotides among which both the previously proved strong enhancer (GNP) and very weak enhancer (ANP) are included. Our results showed that in the presence of phen all of the nucleotides and polyhomonucleotides are strong enhancers of the fluorescence of terbium(III).

The work described here can be divided into two parts: The first part deals with the fluorescence enhancement of terbium(III) by phen including compositional investigations, optimization of the fluorescence output and more detailed studies concerning the effect of pH and of excess reagent, while the second part is concerned with the possibility of the spectrofluorimetric determination of nucleotides as their ternary complexes with terbium(III) and phen.

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Experimental

Apparatus

A Shimadzu spectrofluorimeter, model RF-540 was employed, equipped with a Xenon lamp, a recorder, dual monochromator and a quartz cell (1 × 1 cm cross-section). 10 nm was the slit-width for excitation and emission. Furthermore, a pH meter, model 821 (Zhongshan University, P. R. China) was used.

Reagents

Commercially prepared nucleotides AMP, ADP, ATP, GMP, GDP, GTP (Shanghai Biochemicals, P. R. China) and polyhomonucleotides poly(A), poly(G), poly(C) and poly(U) (Sigma Chemical Co.) were used without further purification; all of the nucleotides and polyhomonucleotides were 5′-phosphates. Their stock solutions were prepared by directly dissolving them in water. Deionized/distilled water was used to prepare the solutions.

A stock solution of terbium(III) (4.0×10^{-3} mol/l) was prepared by dissolving terbium oxide (Tb_4O_7, 99.95% from Beijing Xinxin Chemicals) in conc. HCl and evaporating the solution to dryness. The residue was dissolved in 0.1 mol/l HCl. It was diluted and its pH was adjusted to about 4.5 by 0.1 and 0.01 mol/l sodium hydroxide before use. Phen stock solution (4.0×10^{-3} mol/l) was prepared by dissolving

0.1982 g phen in 20 ml 0.1 mol/l HCl and then diluting to 500 ml with water. More dilute standard solution was prepared by appropriate dilution with water.

For the 0.05 mol/l tris-HCl buffer solution containing 0.1 mol/l sodium chloride, 12.11 g tris, and 11.64 g sodium chloride were dissolved in water and its pH was adjusted by appropriate concentration of HCl and NaOH to a final total volume of 2000 ml.

General procedure

The fluorescence intensities of terbium(III) enhanced by different nucleotides and polyhomonucleotides at neutral pH have been studied in the presence and absence of phen. The influence of the concentration of terbium(III), phen and each nucleotide along with the effect of pH have also been studied.

Typically, samples containing the appropriate concentration of nucleic acid, phen and terbium ion were made up to 10 ml in 5 mmol/l tris-HCl (pH 7.0). All samples were allowed to equilibrate for 5–10 min at room temperature before making fluorescence measurements. Prolonged standing of the samples would lead to a dramatic decrease of the fluorescence intensity. An ionic strength of 10 mmol/l was maintained throughout the experiments. E_x/E_m in the presence and absence of phen is 295.0–298.0/543.5 and 270.0–277.0/492.0 nm, respectively. Fluorescence readings are given as net fluorescence intensities in the arbitrary units of the instrument; background fluorescence has been subtracted for each value reported, except for those in emission spectra.

Results and discussion

Spectral characteristics

The excitation spectra of phen-free and phen-containing solutions prepared according to the procedures described above were studied at pH 7.0 over the wavelength range of 200–400 nm. Upon addition of nucleotides or polyhomonucleotides with phen the excitation spectrum of terbium(III), as monitored from its emission peaks at 492 or 543.5 nm, shifted from a profile characteristic of the rare earth cation (peaks at 235, 265, 355 and 375 nm) to one which represents absorption by the energy transfer complex itself. The spectrum in the presence of phen differs significantly from that in the absence of phen or of the phen-terbium(III) system at the same pH indicating ternary complex formation. The excitation wavelengths in the presence and absence of phen are 295–298 nm or 270–277 nm, respectively. In the excitation spectrum there is a peak at 246 or 272 nm, which is caused by diffraction beam artifacts arising from Rayleigh light scattering by sample molecules, that occur when the excitation spectrum is observed at the emission wavelength of 492 or 543.5 nm.

In all instances the emission spectrum, as depicted in Fig. 1, showed two peaks at 492 and 543.5 nm with excitation range 295–298 or 270–277 nm (Table 1). When the emission spectrum is recorded for 270–277 nm excitation light (in the absence of phen) the emission peak at 543.5 nm is overlapped with the peak caused by the spectrofluorometer's excitation grating monochromator. Therefore, the peak at 492 nm is used for fluorescence intensity measurements, while in the presence of phen with excitation at 298 nm the peak at 543.5 nm is used.

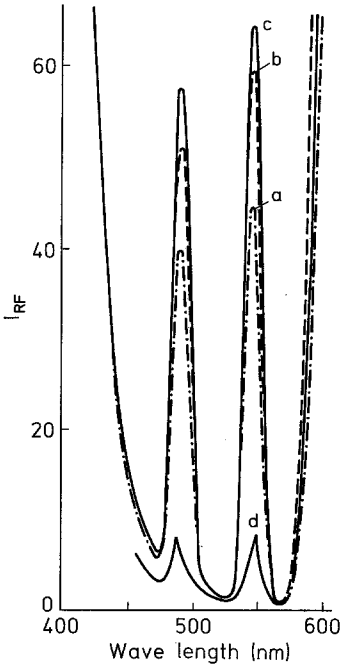

Fig. 1. Emission spectra of combinations of 2.8×10^{-5} mol/l terbium(III), 1.2×10^{-4} mol/l phen and 2.0×10^{-5} mol/l ANP. *a* Tb-phen-AMP; *b* Tb-phen-ADP; *c* Tb-phen-ATP; *d* Tb-phen. The excitation wavelengths were set at 298 nm

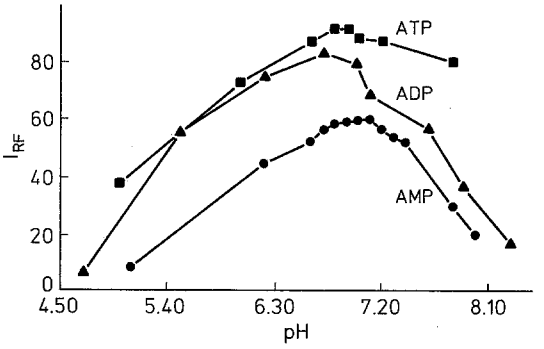

Fig. 2. Effect of pH on terbium(III) emission as its ternary complex with ANP and phen. Conditions: 2.0×10^{-5} mol/l ANP; an ionic strength of 10 mmol/l in sodium chloride; $E_x/E_m = 298/543.5$ nm. AMP: 2.0×10^{-5} mol/l terbium(III) and 8.0×10^{-5} mol/l phen; ADP: 3.2×10^{-5} mol/l terbium(III) and 1.0×10^{-4} mol/l phen; ATP: 8.0×10^{-5} mol/l terbium(III) and 1.6×10^{-4} mol/l phen

Effect of pH

The effect of pH on the terbium(III) emission as its ternary complex with ANP and phen (depicted in Fig. 2) was studied with solutions prepared as described in the procedure. The maximum terbium(III) emission occurs in the pH-range 6.8–7.2 (for GNP the optimal pH was 6.9 to 7.5). The fluorescence decreased at pH greater than 7.2 for ANP, possibly as a result of the formation of insoluble terbium hydroxide [7]; at pH below 4.0 the protonation of the bases prevented the ternary complex formation and the fluorescence diminished.

Effect of the concentration of phen

In the presence of a constant concentration of ANP (2.0×10^{-5}mol/l) the fluorescence intensity reached a

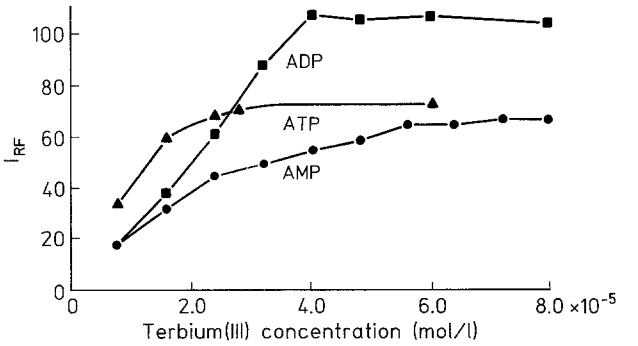

Fig. 3. Effect of terbium(III) concentration on the fluorescence output under 2.0×10^{-5} mol/l ANP at pH 7.0; $E_x/E_m = 298/543.5$ nm. AMP: 8.0×10^{-5} mol/l phen; ADP: 1.0×10^{-4} mol/l phen; ATP: 1.6×10^{-4} mol/l phen

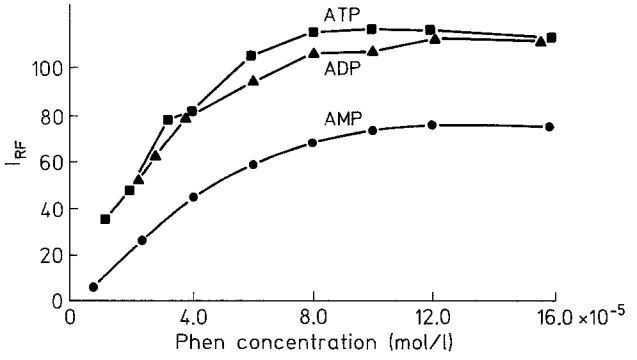

Fig. 4. Effect of phen concentration on terbium(III) emission at pH 7.0. Conditions: 2.0×10^{-5} mol/l ANP; $E_x/E_m = 298/543.5$. AMP: 6.0×10^{-5} mol/l terbium(III); ADP: 2.8×10^{-5} mol/l terbium(III); ATP: 4.0×10^{-5} mol/l terbium(III)

maximum at pH 7.0 when the concentration of terbium(III) was 5.6×10^{-5}, 3.9×10^{-5} and 3.0×10^{-5} mol/l for AMP, ADP and ATP, respectively (Fig. 3). It is obvious, that for the same amount of ANP the saturated concentration of terbium(III) decreased from AMP to ATP, the order of which is opposite to that of the amounts of negative charges on ANP. The ratios of terbium(III) and AMP, ADP or ATP under these conditions are 2.8, 2.0 and 1.5, respectively. The fluorescence intensity was further studied at pH 7.0 as a function of the concentration of phen. The result is presented in Fig. 4. When the concentration of phen increased, the fluorescence of terbium(III) also increased. When it was above two-fold concentration of terbium(III), the fluorescence gradually became constant as depicted in Fig. 4, which suggests a molar ratio of 1:2 for terbium(III) and phen.

Comparison of the fluorescence enhancement of terbium(III) in the presence and absence of phen

The enhancement of terbium(III) fluorescence in the presence and absence of 8.0×10^{-5} mol/l phen was investigated at pH 7.0 with the same concentration of terbium(III) and nucleotides (4.0×10^{-5} mol) or polyhomonucleotides (4.0×10^{-5} mol/l in phosphate residue). Results are presented in Table 1.

From the data presented in this Table the following conclusions are obtained: In the absence of phen the intensities of the terbium(III) fluorescence enhanced by GNP and poly(G) are large, by poly(C) and poly(U) are small, and by ANP and poly(A) are very slight. But, in the presence of phen, all of the nucleotides and polyhomonucleotides studied showed strong enhancement of the terbium(III) fluorescence. The spectrum of this fluorescence is constant at λ_{max} 543.5 and 492 nm, regardless of the nucleotides, though the relative intensity of the fluorescence varies with the nucleotides (Table 1). On the other hand, λ_{max} of the excitation spectra (Table 1) varies somewhat with the nucleotides. This is consistent with the idea that the observed fluorescence enhancement is due to a long-range energy transfer from a particular electronic transition in each nucleotide to the same electronic transition in terbium(III).

With regard to quantitative analysis the fluorescence enhancement of terbium(III) was studied by ANP in the presence of phen. The sensitivity of the fluorimetric determination of ANP has been determined from the slope of the

Table 1. Comparison of terbium(III) fluorescence enhancement by different nucleotides and polyhomonucleotides

Compound	pH	E_x/E_m	Net Fluorescence intensity[a]	Number of determinations
AMP	7.0	298/543.5(270/492)	77(1)[b]	3
ADP	7.0	298/543.5(270/492)	154(1)	3
ATP	7.0	298/543.5(270/492)	135(3)	3
GMP	7.4	298/543.5(270/492)	305(131)	3
GDP	7.4	298/543.5(270/492)	249(212)	3
GTP	7.4	298/543.5(270/492)	61(75)	3
poly(A)	7.0	298/543.5(270/492)	237(3)	3
poly(G)	7.4	295/543.5(270/492)	129(74)	3
poly(C)	7.0	296/543.5(276/492)	195(21)	3
poly(U)	7.0	297/543.5(270/492)	31(7)	3

[a] Fluorescence intensity is given relative to that of AMP in the absence of phen, which is arbitrarily assigned 1; [b] The data in parentheses are values in the absence of phen. Conditions: 4.0×10^{-5} mol/l terbium(III), 8.0×10^{-5} mol/l phen, 4.0×10^{-5} mol/l nucleotides, 4.0×10^{-5} mol/l polyhomonucleotides in phosphate residue except for poly(U) which has a concentration of 1.7×10^{-5} mol/l

Table 2. Analytical parameters of the determination of ANP in the presence of phen

ANP	C_{Tb} (C_{phen}) (μmol/l)	Sensitivity (slope)	Linear range (μmol/l)	LOD (μmol/l)	r	SD (%)
AMP	60(120)	137	0.4–3.0	0.2	0.99936	1.4
		151	30–70		0.99815	1.2
		59	160–280		0.99668	0.2
ADP	80(100)	80	1.0–3.0	0.6	0.99996	0.4
		302	10–40		0.99957	0.7
ATP	80(120)	287	2.0–6.0	1.0	0.99926	0.8
		233	10–30		0.99839	0.3

LOD = Limit of determination; SD = Standard Deviation for 6 measurements using the concentration in the middle of each linear range; r = correlation coefficients. Conditions: pH 7.0, E_x/E_m = 298/543.5 nm

calibration curves of ANP in the presence of phen and the limit of detection from the stability of the blank measurements (Table 2). Though the fluorescence of the system is seriously quenched by proteins, it is quite possible that, with the aid of HPLC, this fluorescence system may be used in quantitative analysis of nucleic acids or nucleotides due to the high sensitivity and reproducibility. Nearly all of the previous reports concerning the analysis of nucleotides by HPLC use EC or UV detectors. A recently published paper [8], reported the use of terbium(III) in the HPLC analysis of a few polyhomonucleotides, among which only poly(G) and poly(X), two strong enhancers, may have practical use in quantitative analysis [8]. In our fluorescene system, however, all polyhomonucleotides and nucleotides studied showed a substantially strong enhancement of the fluorescence of terbium(III), which made this system more attractive in the HPLC analysis of nucleotides and polyhomonucleotides.

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