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Synthesis, characterization and luminescent properties of Eu^{III} and Tb^{III} fluorescent chelates used as label in medical immunoassays

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Abstract

DTPA-pAS, EDTA-pAS, DTPA-pAB and EDTA-pAB are synthesized and confirmed by elemental analysis, IR spectra, ^1H NMR and mass spectra. Fluorescent properties are investigated by mixing the four chelants with Eu^{III}or Tb^{III} chloride in aqueous solution. The fluorescence intensity varies with the chelants, pH value and concentration. Only DTPA-pAS and EDTA-pAS enhance the Tb^{III} luminescence efficiently and the fluorescence intensity varied slightly at pH 6.0–12.0. The fluorescence of Tb^{III}-DTPA-pAS can be detected even at a concentration of 10^{-7} mol l^{-1}. Tb^{III}-DTPA-pAS has been used as a label in fluoroimmunoassay for the determination of human serum albumin (HSA).

1. Introduction

Eu^{III} fluorescent chelates can be used as labels in immunoassays [1,2], but until now, the most successful label has been an essentially non-fluorescent Eu^{III} chelate, such as Eu^{III} with DTPA. The Eu^{III} ions have to be extracted subsequently into an enhancement solution to sensitize the fluorescence. Bailey found that a Tb^{III} chelate with DTPA-pAS was intensely fluorescent, its fluorescence was relatively insensitive to the variation of pH and the chelate was stable even in highly dilute solution. He used the chelate solution for labelling the sample in a HSA fluorescent immunoassay [3], but he had not isolated either the chelant or the Tb^{III} chelate [4].

We now report the synthesis of the four chelants, DTPA-pAS, EDTA-pAS, DTPA-pAB and EDTA-pAB, the sensitization for the luminescence of Eu^{III} and Tb^{III} in aqueous solution by the four chelants, and the use of Tb^{III} chelate with DTPA-pAS as label in fluoroimmunoassay for the determination of HSA.

2. Experimental details

2.1 Apparatus and measurements

C, H, N, analyses were performed on a Perkin–Elmer 240-C analyzer. IR measurements were made on a Nicolet FTIR-5DX instrument with a 4 cm^{-1} resolving power using KBr pellets. Spectra were recorded from 400 to 3600 cm^{-1} and the scanning speed was about 0.32 cm^{-1} s^{-1}. ^1H NMR spectra were determined with a JEOL FX-90Q model spectrometer at room temperature with $(CD_3)_2SO$ as solvent. Positive ion FAB mass spectra were obtained on a ZAB-HS mass spectrometer using a glycerol matrix and xenon gas. Fluorescence emission and excitation spectra were determined on a Hitachi absolute spectrofluorophotometer model 850.

2.2. Synthesis of chelants

Diethylenetriaminepentaacetic acid anhydride (DTPAA) or ethylenediaminetetraacetic acid anhydride (EDTAA) and sodium *p*-aminosalicylate (pASNa) or sodium *p*-aminobenzoate (pABNa) mixed in a 1:1 mole ratio in DMSO at room temperature, after stirring for 5 h, water was added and stirring was continued for 2 h. The products were isolated, washed with ethanol and dried at 90 °C. The dried products were re-precipitated from aqueous solution by treating with NaOH and H_2SO_4.

2.3. Studies of fluorescent properties

Eu^{III} or Tb^{III} chloride and a chelant were mixed in 1:1 mole ratio in water. Fluorescence of the mixture was measured.

3. Results and discussion

3.1. Synthesis and characterization of chelants

The four chelants were synthesized as shown by the main reaction in Fig. 1. The other three chelants were synthesized by replacement of EDTAA for DTPAA

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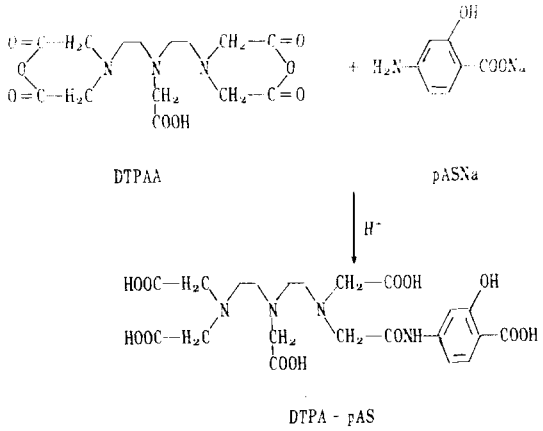

Fig. 1. Main reaction of the four synthesized chelates.

TABLE 1. Elemental analysis for chelants

Chelant	C%		H%		N%	
	Exp	Calc	Exp	Calc	Exp	Calc
DTPA-pAS	46.86	46.15	5.46	5.49	9.67	10.26
DTPA-pAB	49.00	48.74	5.55	5.42	10.09	10.83
EDTA-pAS	46.15	45.84	4.73	5.17	9.09	9.44
EDTA-pAB	46.55	47.55	5.15	5.36	9.46	9.79

and pAB for pAS. The products were confirmed by elemental and MS analysis, and also characterized by IR and ^{1}H NMR spectra. The experimental results are shown in Tables 1–3.

As shown in Table 1, the results of the elemental analysis are close to the calculated values. Each molecular ion appears in the fragmentation process for each chelant. (Table 2). The IR spectra of the four chelants both contain ν(C=O) bands in the range 1664–1697 cm^{-1}, which were assigned to the absorption of –COOH and –CONHR. In the range 1537–1554 cm^{-1}, there are medium bands, which were characterized as the vibration δ(N–H) in secondary acylamide. The existence of the –CONHR group for the four chelants was also confirmed by ^{1}H NMR spectra as shown in Table 3. There are proton bands at ~7.7 ppm, which were attributed to the –CONHR group.

3.2. *Fluorescent properties of Eu^{III} and Tb^{III} with chelants in aqueous solution*

The data in Table 4 show that the four chelants can enhance Tb^{III} luminescence in aqueous solution. The sequence of enhancement is EDTA-pAS > DTPA-pAS > DTPA-pAB > EDTA-pAB. The four chelants, however, yield almost no enhancement of the Eu^{III} luminescence. Table 5 indicates that the fluorescence intensity varied only slightly over the pH range 6.0–12.0 and the fluorescence intensity decreased notably at pH 4.0. This demonstrates that the chelates of DTPA-pAS and EDTA-pAS with Tb^{III} are stable in alkaline solution, but dissociate in acidic solution. Table 6 shows that the fluorescence of Tb^{III}-DTPA-pAS can be detected even at the concentration of 10^{-7} mol l^{-1}, while, Tb^{III}-EDTA-pAS luminescence decreases evidently as the concentration lowered. This may be due to the fact

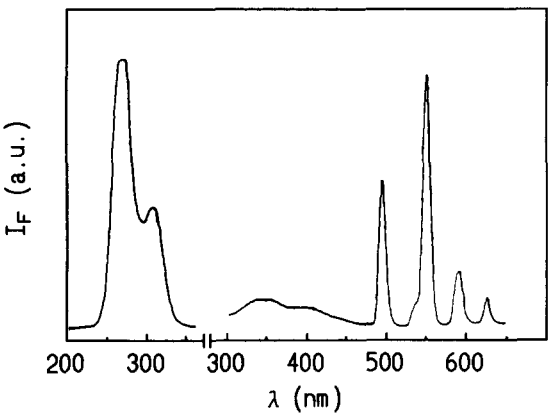

Fig. 2. Excitation and emission spectra of HSA-DTPA-pAS-Tb^{III}.

TABLE 2. Mass spectral analysis for chelants

Chelant	DTPA-pAS	EDTA-pAS	DTPA-pAB	EDTA-pAB
Peak (M+1)	529	428	513	412

TABLE 3. Chemical shifts (ppm) and assignments for ^{1}H NMR of the chelants

DTPA-pAS	EDTA-pAS	DTPA-pAB	EDTA-pAB	Assignment
2.4–3.3	2.4–3.2	2.5–3.0	2.5–3.0	$-CH_2-$
3.4–3.9	3.4–3.9	3.5–3.6	3.4–3.8	R_2NCH_2CO-
6.6–7.1	7.0–7.1	–	–	Ar–OH
7.3	7.3	7.6	7.3	Ar–H
7.6–7.7	7.6–7.7	7.8–7.9	7.6–7.9	–CONHAr
10.2	10.2	10.3	10.3	–COOH

TABLE 4. Emission spectra data for Eu^{III} and Tb^{III} chelates in aqueous solution (1×10^{-3} mol l^{-1}, pH 7.0)

Chelant	RE^{III}	λ_{ex} (nm)	λ_{em} (relative intensity) (nm)			
DTPA-pAS	Eu^{III}	–		–		
	Tb^{III}	333	489(14.9)	546(30.0)	586(6.1)	621(3.3)
EDTA-pAS	Eu^{III}	299	401(0.16)	599(0.01)	–	
	Tb^{III}	350	491(33.4)	547(103.6)	586(14.6)	621(7.0)
DTPA-pAB	Eu^{III}	296	336(0.08)	595(0.10)	616(0.24)	695(0.07)
	Tb^{III}	302	489(6.3)	546(10.7)	586(2.4)	621(1.3)
EDTA-pAB	Eu^{III}	300	338(1.93)	616(0.14)	658–670(0.2)	
	Tb^{III}	228	489(1.3)	546(2.5)	586(0.5)	621(0.3)

TABLE 5. The influence of pH value on $^5D_4\rightarrow{}^7F_5$ emission band data for Tb^{III} chelates (1×10^{-4} mol l^{-1})

System	pH value	λ_{ex}	λ_{em} (relative intensity)
Tb^{III}-DTPA-pAS	4.0	301	546(9.9)
	6.0	304	546(33.7)
	8.0	302	546(37.0)
	10.0	302	546(37.5)
	12.0	299	546(37.3)
Tb^{III}-EDTA-pAS	4.0	257	547(5.7)
	6.0	327	547(49.9)
	8.0	331	547(60.8)
	10.0	330	547(59.3)
	12.0	330	547(56.8)

TABLE 6. The influence of concentration on emission spectrum data (pH 7.0)

System	Conc. (mol l^{-1})	λ_{ex} (nm)	λ_{em} and relative intensity (nm)				
Tb^{III}-DTPA-pAS	10^{-7}	264	408(0.02)	491(0.13)	547(0.22)	587(0.05)	622(0.03)
	10^{-6}	263	408(0.10)	489(1.50)	546(2.57)	586(0.60)	621(0.30)
	10^{-5}	263	408(0.60)	489(11.4)	546(19.3)	586(4.3)	621(2.3)
	10^{-4}	303	408(1.54)	489(25.9)	546(43.6)	586(9.7)	621(5.2)
	10^{-3}	335	–	489(20.0)	546(38.5)	586(7.5)	621(4.2)
Tb^{III}-EDTA-pAS	10^{-7}	263	408(0.03)	–			
	10^{-6}	261	401(0.26)	490(0.05)	546(0.07)	586(0.01)	621(0.01)
	10^{-5}	266	401–408(1.30)	489(5.1)	547(15.6)	586(2.3)	621(1.1)
	10^{-4}	328	–	491(22.1)	547(65.0)	587(9.2)	622(4.5)
	10^{-3}	351	–	491(30.7)	547(94.2)	587(13.52)	622(6.6)

that chelation of DTPA-pAS with Tb^{III} is more stable than that of EDTA-pAS. From 10^{-5} to 10^{-3} mol l^{-1}, the λ_{ex} value changed obviously. This may be caused by the formation of a bipolymer of chelates.

3.3. Tb^{III} chelate with DTPA-pAS as label in fluoroimmunoassay for the determination of HSA

Procedures for labelling the Tb^{III} chelate of DTPA-pAS with HSA were the same as that described in the literature [3]. Fluorescence emission and excitation spectra are displayed in Fig. 2. The peaks at 266 and 306 nm are the excitation spectrum of the label. The weak and broad peak from 300 to 460 nm results from the protein and the ligand. The sharp peaks at 489, 546, 586 and 621 nm are the characteristic emission of Tb^{III}. The specific activity of the label was calculated from the ratio of concentration of Tb^{III} and HSA in the label, [Tb^{III}]/[HSA], and found to be 1.4.

Figure 3 shows the standard curve of HSA. B and B_0 represent the relative fluorescence intensity of the sample and maximum binding, respectively. The range of the standard curve is 0.08–5.0 mg ml^{-1}. This result

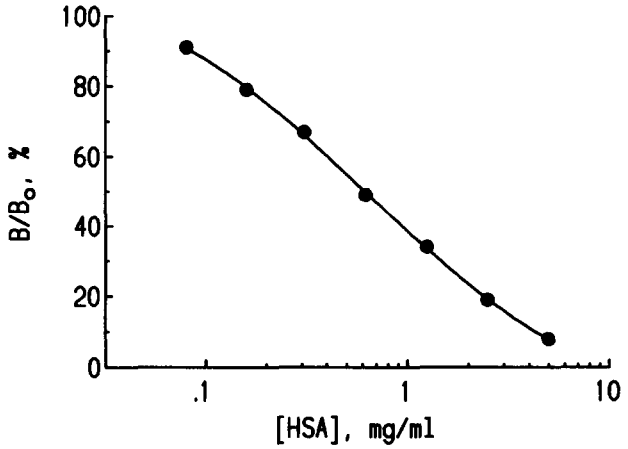

Fig. 3. B/B_0-[HSA] standard curve.

shows that using the chelate Tb^{III}–DTPA-pAS as label, the fluorescence intensity of the immunoreactive complex can be measured directly. Therefore, the chelant that we prepared can be used in Tb^{III} ion fluorescence immunoassays.

4. Conclusion

The chelants studied here possess the following advantages:

(1) chelates with Tb^{III} ion are stable and can dissolve in water easily;

(2) sensitizing Tb^{III} luminescence intensely;

(3) the fluorescent immunoreactive complex can be measured directly, without addition of an enhancing solution.

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