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Fluorescence study of some terbium–oligopeptide complexes in methanolic solution

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Abstract

This study concerned the use of lanthanide chelates to detect glycyl-leucyl-phenylalanine (GLF) and its homologues. Spectroscopic analysis of peptides without or with terbium complexation revealed the formation of $(LF)_3(Tb)_2$, $(GF)_3(Tb)_2$, $(GLF)_3(Tb)_2$ and $(FL)_4Tb$, $(FG)_4Tb$ complexes with high stability constants in methanolic solutions ($pK_d > 13$). Lanthanide chelate emission displayed a large Stokes shift (> 270 nm), which allowed Tb chelates of GLF and its derivatives to be used for detection purposes. However, this preliminary study indicated some important limitations associated with lanthanide chelation, such as high methanolic content.

Keywords: Oligopeptides; Terbium; Complexes; Luminescence

1. Introduction

Oligopeptides are of increasing interest in academic research and the pharmaceutical industry [1–4]. Glycyl-leucyl-phenylalanine (GLF), which has been isolated from human milk proteins [5–7], possesses immunostimulation properties. For potential pharmacological uses, GLF and its related compounds, glycyl-phenylalanyl-leucine (GFL), phenylalanyl-glycine (FG), glycyl-phenylalanine (GF), phenylalanyl-leucine (FL) and leucyl-phenylalanine (LF), need to be detected at a low concentration in various environments (hydrolysates, biological matrices, etc.). As these compounds possess the phenylalanine chromophore, they can be detected by absorbance as well as direct fluorimetry [8]. However, as the chemical structure of oligopeptides induces complexation with metals of analytical interest, such as Cu(II) and Ni(II) [9–15], we focused our study on the possible use of lanthanide chelates as a detection mode for these peptides. Lanthanide chelates, because of their luminescence properties

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and potential use in a variety of chemical and biological applications [16,17], have attracted considerable interest recently. To our knowledge, the present study is the first to investigate lanthanide chelation of GLF and its derivatives in this context.

2. Experimental

2.1. Chemicals

All peptides were in levogyral (natural) form and supplied by Bachem Biochimie S.A.R.L. (Voisin-le-Bretonneux, France). Tris(hydroxymethyl)-aminomethane, sodium acetate, sodium borate, terbium chloride and europium chloride were of analytical-reagent grade and obtained from Sigma (St. Quentin-Fallavier, France). Methanol (BDH Laboratory Supplies, Poole, UK) and water (Biosedra, Louviers, France) were of high-performance liquid chromatograpy (HPLC) grade and carefully checked to ensure that there were no fluorescence impurities.

2.2. Apparatus and method

Absorbance measurements were performed on a UV–visible Shimadzu UV160 model spectrophotometer (Touzart and Matignon, Courtaboeuf, France) equipped with a 10-mm optical pathlength quartz cuvette. The luminescence spectrometer was a Perkin–Elmer LS50B model (Montigny-le-Bretonneux, France). The source was a pulsed xenon flash lamp with a pulsewidth at half peak height of 8 μs and power equivalent to 20 kW. Flashes were produced with a 20 ms cycle. All spectroscopic measurements (absorbance (A), direct fluorescence (F) and time-resolved fluorescence (TRF)) were performed in triplicate on methanolic solutions at room temperature (20 ± 2°C).

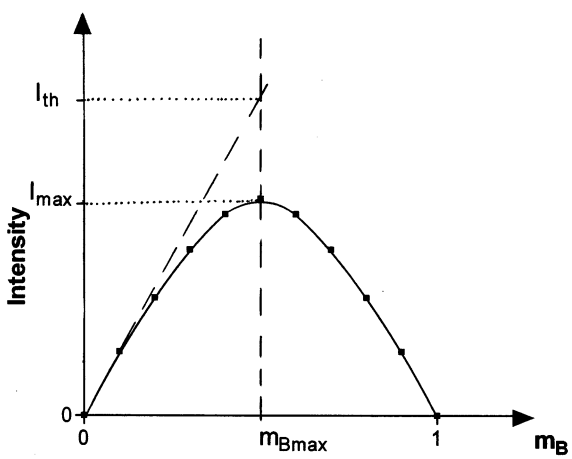

Fig. 1. Theoretical Job's curve.

2.3. Computerization

The continuous variation method (known as Job's curve) was used to define the stoichiometry of the complexation reaction and the stability constant [18]. This method consists in measuring the fluorescence signal for different mixtures of terbium and ligand. A fixed total concentration (the sum of the concentrations of all components) is used, and the terbium concentration is either increased (with a corresponding decrease in peptide concentration) or decreased (with a corresponding increase in peptide concentration).

For Eq. (1), the constant K_S can be calculated from Job's curve (Fig. 1) according to the relation in Eq. (2):

$$x\mathrm{A} + y\mathrm{B} \overset{K_S}{\leftrightarrows} \mathrm{A}_x\mathrm{B}_y \quad \text{with} \quad K_S = \frac{[\mathrm{A}_x\mathrm{B}_y]}{[\mathrm{A}]^x[\mathrm{B}]^y} \tag{1}$$

$$K_S = \left(\frac{x+y}{C_A + C_B}\right)^{(x+y-1)} x^{-x} y^{-y} \gamma (1-\gamma)^{-(x+y)} \tag{2}$$

where m_B is the mole fraction of B, $C_A + C_B$ is a constant, and C_A and C_B are concentrations of A and B, respectively, in the solution, x and y are determined graphically from the maximum position on the curve,

$$m_{B_{max}} = \frac{y}{x+y}$$

$$\gamma = \frac{I_{A_xB_y}}{I_{th}} \quad \text{for } m_B = m_{B_{max}}$$

where $I_{A_xB_y}$ is the emission intensity of the complex in solution (partially dissociated) and I_{th} is the emission intensity of the complex if not dissociated (theoretical).

Table 1
Absorbance measurement (RSD%) for the studied peptides and their mixtures with terbium (1:1 or stoichiometric ratio)

	λ to measure (nm)	ε (RSD%) (l mol^{-1} per cm)
LF (10^{-3} M)	255	190 (3.6)
$(LF)_3(Tb^{3+})_2$ (2×10^{-4} M)	255	1195 (2.9)
LF:Tb^{3+} 1:1 (5×10^{-4} M)	259	(A/lC) 216(7.9)
FL (10^{-3} M)	255	182 (4.2)
$(FL)_4(Tb^{3+})$ (2×10^{-4} M)	255	1277 (2.4)
FL:Tb^{3+} 1:1 (5×10^{-4} M)	259	(A/lC) 214 (8.5)
GF (10^{-3} M)	255	188 (2.8)
$(GF)_3(Tb^{3+})_2$ (2×10^{-4} M)	255	933 (2.4)
GF:Tb^{3+} 1:1 (5×10^{-4} M)	259	(A/lC) 215 (6.4)
FG (10^{-3} M)	255	171 (1.2)
$(FG)_4(Tb^{3+})$ (2×10^{-4} M)	255	1068 (9.0)
FG:Tb^{3+} 1:1 (5×10^{-4} M)	259	(A/lC) 202 (7.7)
GLF (5×10^{-4} M)	255	185 (3.2)
$(GLF)_3(Tb^{3+})_2$ (1×10^{-4} M)	255	800 (12.5)
GLF:Tb^{3+} 1:1 (3.33×10^{-4} M)	259	(A/lC) 211 (0.8)
GFL (2.5 10^{-4} M)	255	184 (7.5)
Not determined		
GFL:Tb^{3+} 1:1 (1.25×10^{-4} M)	259	(A/lC) 288 (11.8)

I_{th} can be graphically estimated by the intersection of tangentials at each extremity. This method, though commonly used, can lead to error, especially when x and y are very different. To minimize error, the intersection of the curve tangential (on the maximum side) and the perpendicular at $m_{B_{max}}$ were considered (see Fig. 1).

If A_xB_y is not dissociated, m_A moles of A react with $m_{B_{max}}$ moles of B to yield $1/(x+y)$ moles of A_xB_y. Thus, for $m_B = m_{B_{max}}$, $I_{max} = I_{th} = I_{A_xB_y}$. In reality, as the complex can be dissociated ($I_{A_xB_y}/I_{th} = \gamma < 1$), only $\gamma/(x+y)$ moles of A_xB_y are in solution, i.e. γm_B moles of B is involved in the reaction and $(1-\gamma)m_B$ moles is free in the solvent.

The K_S unit depends on the x and y parameters and can be expressed as $(\text{mol l}^{-1})^{1-x-y}$. It can be define a dissociation constant $K_d = 1/K_S$, preferentially used as $pK_d = -\log K_d = \log K_S$ [19]. In this case, no units are required, and pK increases when complex stability increases.

3. Results and discussion

3.1. Spectroscopic measurements

The main purpose of this study was to consider the feasibility of terbium chelation by oligopeptides analogous to GLF and then to apply this property for detection after chromatographic separation of the peptides. Methanolic solutions, which are routinely used in HPLC, ensured a perfect solubility of the free and complexed species studied and eliminated the quenching effect of water [16].

The luminescence detector was set at $\lambda_{exc} = 258 \pm 1$ nm. For TRF, delay time (td) and gate time (tg) were optimized at 0.1 and 5 ms, respectively. To observe possible modifications in the absorption and emission spectra of the studied peptides without or with terbium complexation, data were recorded first for peptide (P) alone and with a P:Tb^{3+} ratio of 1. The P:Tb^{3+} mixture was then studied using a stoichiometric ratio determined as indicated below (except for GFL, for which more than one complex appeared). The very high stability constants found (see Table 4) at the stoichiometric ratios used in this study gave essentially no uncomplexed species in the solution, which is why the mixture with a stoichiometric ratio of oligopeptide and terbium was considered as a pure solution of the corresponding complex. For the terbium solution, absorption at 258 nm was negligeable, and the emission at 545 nm ($\lambda_{ex} = 258$ nm) was weak, as indicated in Section 3.3. The excitation spectra recorded for each mixture showed that a similar λ_{exc} (258 nm) induced maximum emission spectra at 545 nm. The results for absorbance (A), direct fluorescence (F) and time-resolved fluorescence (TRF) are summarized in Tables 1 and 2 and discussed further on. The

Table 2
Fluorimetric measurements (RSD%) for the studied peptides and their stoichiometric mixture, at $\lambda_{ex} = 258$ nm (for TRF, td = 0.1 ms, tg = 5 ms)

	F (286 nm)			F (545 nm)				FRT (545 nm)			
	I_F	C (10^{-5} M)	I_F/C (10^5 M^{-1})	I_F	I_F/I_A	C (10^{-5} M)	I_F/C (10^5 M^{-1})	I_F	I_F/I_A	C (10^{-5} M)	I_F/C (10^5 M^{-1})
FL	673 (1.9%)	8	84	–	–	–	–	–	–	–	–
$(FL)_4Tb$	625 (2.2%)	2	313	81 (3.3%)	0.99 (1.9%)	20	4	340 (3.8%)	0.99 (2.3%)	2	170
FG	588 (2.3%)	8	74	–	–	–	–	–	–	–	–
$(FG)_4Tb$	560 (1.2%)	2	280	41 (3.1%)	1.01 (1.7%)	20	2	381 (3.9%)	1.02 (2.8%)	2	191
LF	737 (2.4%)	6	123	–	–	–	–	–	–	–	–
$(LF)_3(Tb)_2$	547 (1.6%)	2	273	310 (0.7%)	0.96 (1.2%)	20	16	750 (4.5%)	1.00 (1.2%)	2	375
GF	849 (0.1%)	6	142	–	–	–	–	–	–	–	–
$(GF)_3(Tb)_2$	581 (1.4%)	2	290	301 (0.8%)	0.97 (0.9%)	20	15	589 (5.9%)	0.91 (2.0%)	2	295
GLF	752 (4.9%)	6	125	–	–	–	–	–	–	–	–
$(GLF)_3$ $(Tb)_2$	530 (2.1%)	2	265	196 (2.1%)	0.98 (0.5%)	10	20	703 (1.7%)	0.95 (1.6%)	2	352

typical spectra of TRF in the case of LF are presented in Fig. 2

3.2. Absorbance study

All studied peptides showed non-specific transition, with λ_{max} located in the 211–218 nm range and a shoulder at 259 nm as a result of the electron-π of the aromatic ring of the phenylalanine. At this wavelength, the A/l.C ratio was determined for peptide alone and for the 1:1 mixture of peptide and $TbCl_3$ in order to evaluate terbium chelation of the peptide. Obviously, this ratio corresponded to the molar absorption coefficient (ε) for peptide alone, but not for the 1:1 mixture which contained free peptide, free terbium and chelate, as indicated by the stoichiometric calculations and the stability constants (see Section 3.4). The absorption spectrum of a methanolic 10^{-3} M solution of $TbCl_3$ displayed

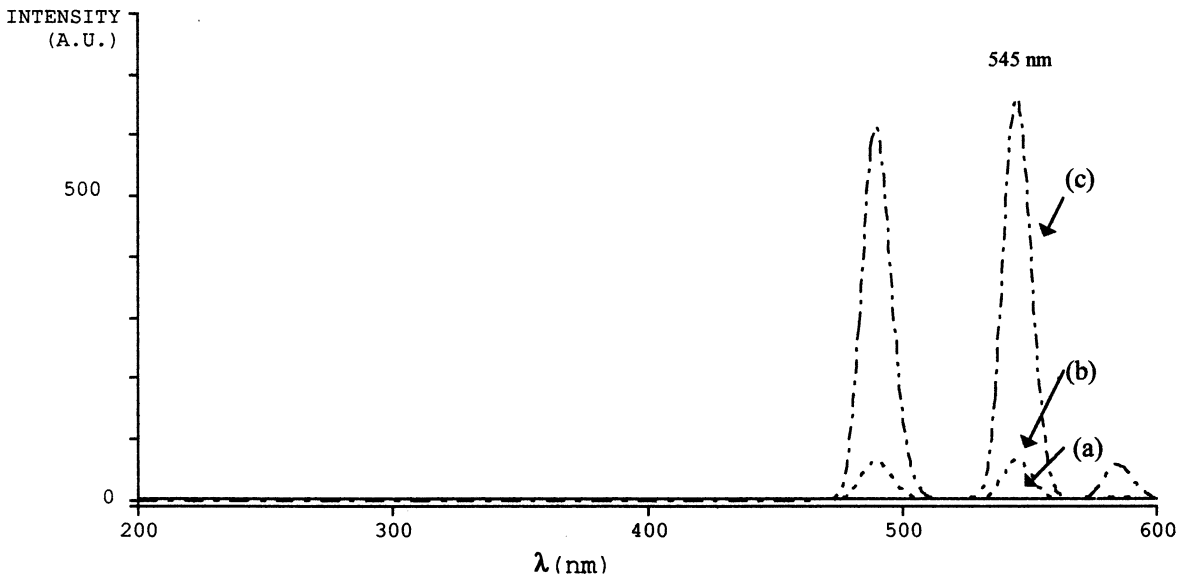

Fig. 2. Time resolved fluorescence spectra ($\lambda_{ex} = 258$ nm) in methanolic solutions: (a) LF 6×10^{-5} M; (b) $TbCl_3$ 4×10^{-5} M; (c) $(LF)_3(Tb)_2$ $2. \times 10^{-5}$ M.

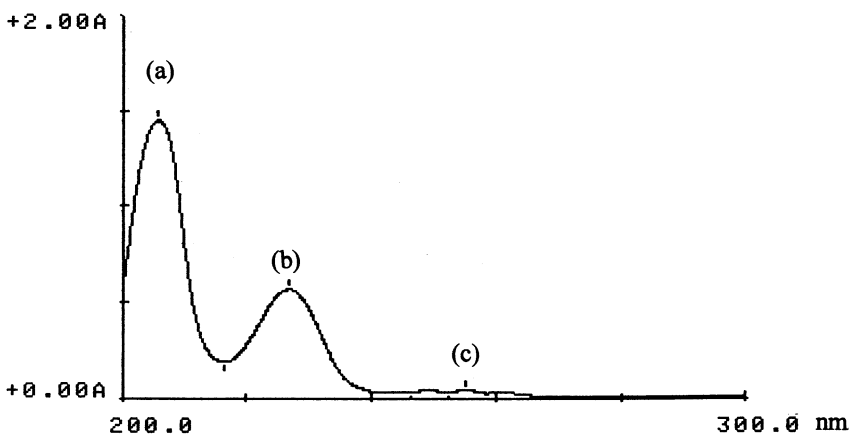

Fig. 3. Absorbance spectra of $TbCl_3$ 10^{-3} M in methanolic solutions: (a) 207 nm; (b) 227 nm; (c) 258 nm.

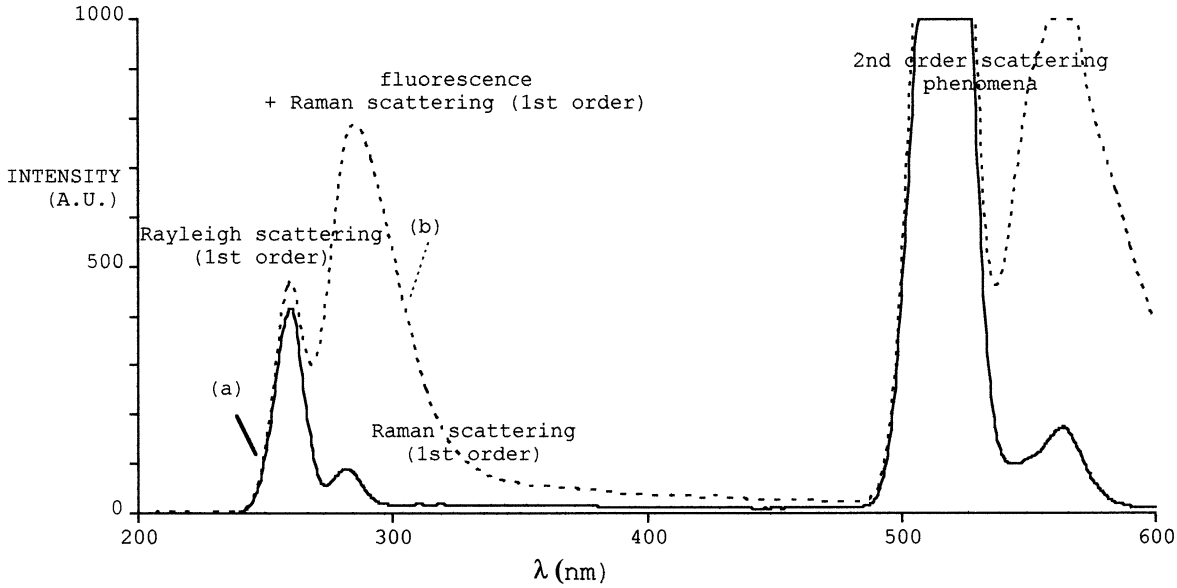

Fig. 4. Fluorescence emission spectra ($\lambda_{ex} = 258$ nm) in methanolic solutions: (a) LF 6×10^{-5} M; (b) MeOH.

very weak absorbance at 258 nm (< 0.05), as shown in Fig. 3.

Thus, the observed increase in the ratio value between peptide alone and a 1:1 mixture with $TbCl_3$ (Table 1) indicated that complexation had occurred (absorption was much greater than with the corresponding peptide alone). Table 1 confirms this increase in the molar absorption coefficient, showing a factor in the 4–7 range from free peptide to complexes, with no λ_{max} displacement.

3.3. Fluorimetric (F) and time-resolved fluorimetric (TRF) spectroscopy

As shown in Fig. 4, peptide fluorescence attributable to the phenylalanyl residue was observed at 286 ± 2 nm. However, the usual interference phenomena of Rayleigh and Raman scattering were clearly visualized on the spectrum [20,21]. As Raman scattering of methanol occurred simultaneously with fluorescence emission, this property could not be used for the assay.

Concerning Tb:P complexes (Fig. 5), residual emission at 286 nm showed that energy was not completely transferred to the terbium ion, even for the stoichiometric ratio. Because of a second-order scattering phenomenon in the 500–600 range, a 390 cut-off emission filter had to be used to observe emission at 545 nm (Fig. 5b). TRF emission was seen at the same time (Fig. 5c)

For convenience, the ratio of total fluorescence intensity to absorption intensity (each in arbitrary unit) at maximum excitation (I_F/I_A), is shown in Table 2 for the stoichiometric ratio. Energy transfer was found to be similar for all complexes.

Table 2 also compares the ratio of fluorescence intensity (arbitrary unit) to the molar concentration of the solution (I_F/C) for excitation at 258 nm. For I_F at 286 ± 2 nm, the Raman scattering phenomenon interfered with fluorescence measurements. Even though the reported value took subtraction of pure methanol Raman scattering into account, it was only approximate since scattering phenomena varied when solutes were dissolved in the solvent. Given this approximation, fluorescence (I_F) for the studied oligopeptide alone was higher when phenylalanine was C-terminal rather than N-terminal. In the complex, fluorescence at 286 ± 2 nm (resulting from peptide) was higher than (F) at 545 ± 2 nm (resulting from terbium). On the other hand, lanthanide chelate emission at 545 nm, characterized by a

large Stokes shift (> 270 nm), indicated that terbium chelates are of interest for detection of the studied oligopeptides. TRF measurements corresponding to an integration on 5 msec rather than a punctual value for F were indicative of increasing factors from F to TRF, i.e. 100 for FG, 40 for FL and 20 for LF, GF and GLF, responsible for better sensitivity. Moreover, two groups of oligopeptides can be defined, one containing N-terminal phenylalanine and exhibiting lower TRF intensity than the other with C-terminal phenylalanine.

In a first approximation, a metal chelate with an aromatic ligand can be regarded as possessing ligand-localized and metal-centered orbitals [22]. Absorption of a photon may cause an electron to be promoted from a ligand-localized orbital to a metal-centered orbital, or vice-versa, in which case the transition is designated as an 'intramolecular charge-transfer' transition [23]. Fig. 6 provides a schematic level diagram depicting the probable mechanism of de-excitation in the complex. Excitation in the near-ultraviolet region (258 nm) induced singlet-singlet absorption transition into the peptide. Two de-excitation pathways seemed to develop simultaneously: one due to native ligand fluorescence at 286 nm and the other due to terbium fluorescence at 545 nm, which could be determined directly or time-resolved. It is noteworthy that intramolecular energy transfer occurred only when the lowest triplet level of the ligand at higher energies was close to the resonance levels of the lanthanide ion. In the same conditions, the use of europium chloride instead of terbium did not produce radiative emission, probably because of too great a difference in energy levels.

Table 3 compares the intensity I_F for each type of emission in the case of $(LF)_3(Tb)_2$. It is apparent that TRF is preferable because scattering phenomena cannot interfere when an appropriate choice of td is made (= 0.1 ms) and the sensitivity is better, which confirms the potential interest of these complexes as derivatized compounds for further detection of peptides. Although terbium showed no significant absorption at 258 nm, an

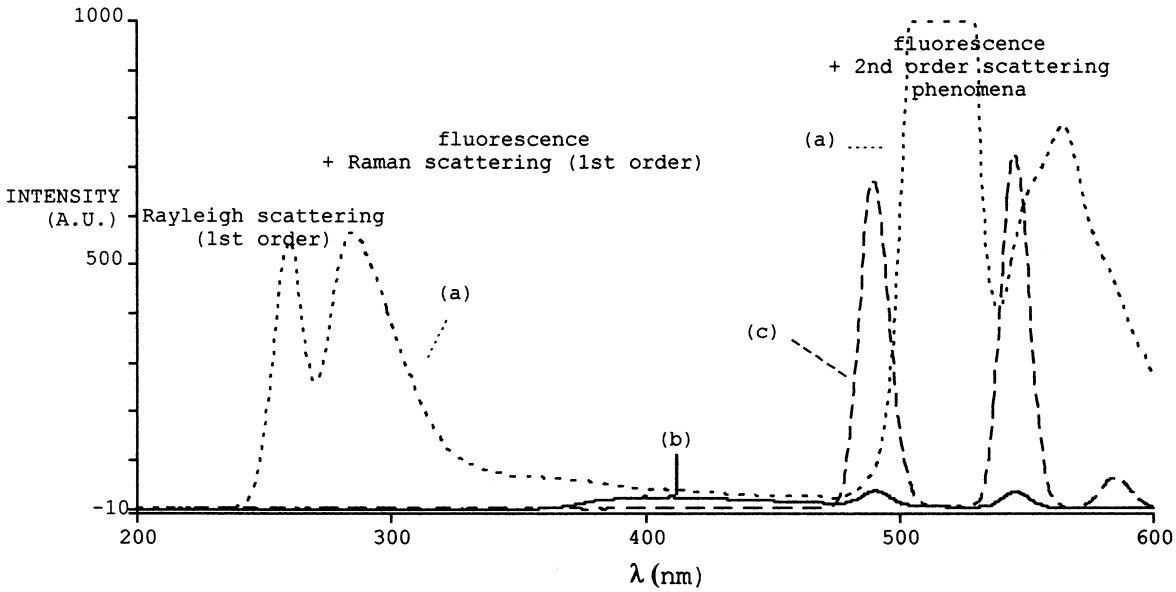

Fig. 5. Fluorescence emission spectra of $(LF)_3(Tb)_2$ ($\lambda_{ex} = 258$ nm), 2×10^{-5} M in methanolic solutions: (a) without an emission filter (F); (b) with an emission filter 390 cut-off (F); (c) with td = 0.1 ms, tg = 5 ms (TRF).

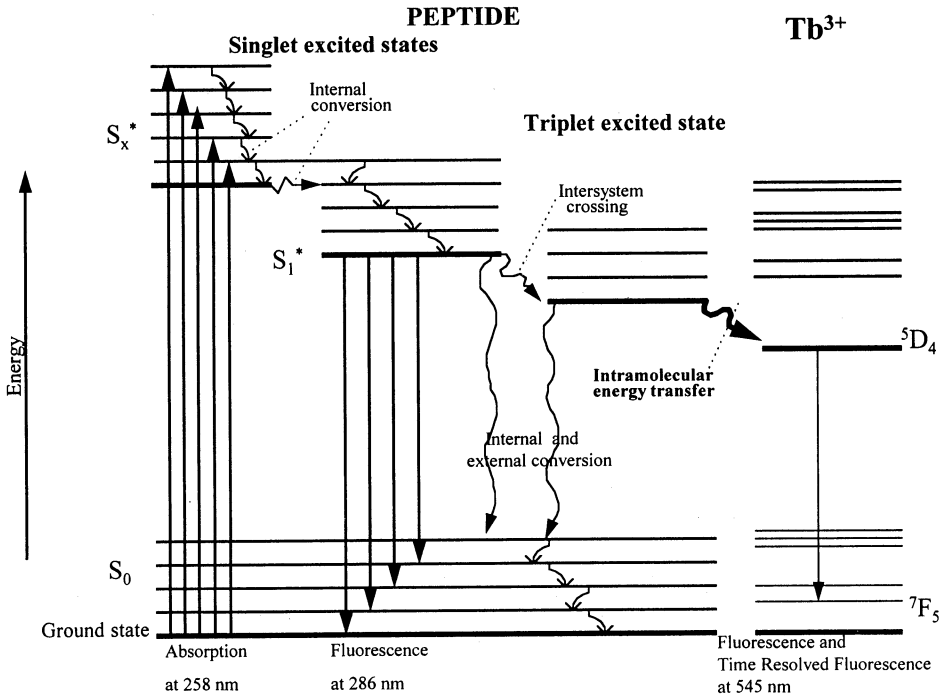

Fig. 6. Proposed general pathway of terbium chelate energy transfer.

Table 3
Comparison of I_F by F (286 nm), F (545 nm), TRF (545 nm) for $(LF)_3(Tb)_2$ 2×10^{-5} M and corresponding concentrations of LF and Tb^{3+}

	Concentration (M)	I_F(F) 286 nm	I_F(F) 545 nm	I_F(FRT) 545 nm
Scattering phenomena		Substraction of pure methanol Raman scattering	No interference when using a 390 nm cut-off filter at emission	No interference when using td = 0.1 ms
$(LF)_3(Tb)_2$	2×10^{-5}	494	Not detected	750
	2×10^{-4}	Saturated	310	Saturated
LF	6×10^{-5}	730	0	0
Tb	4×10^{-5}	0	Not detected	80

emission was always recorded for excitation at this wavelength. When this phenomenon was studied in the $0–10^{-4}$ M range, luminescence response was found to be linear at 545 nm for $\lambda_{exc} = 258$ nm in terms of the equation $I_{Tb} = 18.2C_{Tb} + 6.8$, with a correlation coefficient of 0.9985. The initial ordinate was significantly different from zero, which can be attributed to the formation of methoxy species in methanolic solution [24].

3.4. Stoichiometry and stability constants

Lanthanide complexation reactions [16] in aqueous solutions are assumed to proceed by a mechanism involving initial formation of an intermediate outer sphere species in which the lanthanide (cation) and the ligand (anion) are separated by one or more intervening molecules of hydrate water. The expulsion of this water leads to the formation of an inner sphere complex

in which the anion and cation are in direct contact. Some ligands cannot displace the water, and complexation terminates with the formation of the outer sphere species. Lanthanide cations have been found to form both inner and outer sphere complexes, and for some ligands both types of complexes may be present simultaneously. However, it is likely that Ln^{3+} cations that ligands whose pK_a values are <2 form predominantly an outer sphere complex, while those for which $pK_a > 2$ form predominantly an inner sphere complex. On this basis, we have proposed Eq. (3) with an inner sphere complex because studied peptides present $pK_a > 2$ (see Table 4), terbium is a trivalent lanthanide cation and methanol a polar solvent with a hydroxyl group able to complex lanthanide [23] as described for water.

In Section 2.3, a theoretical interpretation of Job's curves is given for a simple case in which there is only A_xB_y and not A or B emission: $I_{max} = I_{A_xB_y}$. As our experiments needed to take into account the slight fluorimetric response of terbium (B) when alone in the solution, emission I_{max} corresponded to the sum of chelate emission and free terbium emission.

$$xP + yTb \rightleftarrows P_xTb_y \quad (3)$$

Hence,

$$I_{max} = \gamma I_{th} + (1-\gamma)I_{Tb}$$

$$I_{max} = \gamma(I_{th} - I_{tb}) + I_{Tb}$$

thus,

$$\gamma = \frac{I_{max} - I_{Tb}}{I_{th} - I_{Tb}}$$

For instance, Fig. 7A shows the Job's curve obtained for LF ($n = 6$).

Table 4 summarizes the experimental conditions used for all peptides and the x, y and pK_d values found for each studied peptide.

The same two groups of oligopeptides can be redefined, one showing a pK_d close to 18 and C-terminal phenylalanine, and the other a pK_d close to 14 and N-terminal phenylalanine. In fact, the lanthanide cation behaves in their complexes like a typical hard acid, interacting preferentially

Table 4
pK_a (in water) of the free carboxyl function in peptides[a]

Peptide	pK_a of free carboxyl	Factor m_{Tb} (M)	x	y	pK_d (ΔpK_d)
FL	2.36	5×10^{-4}	4	1	14.9 (0.8)
FG	2.34	10^{-3}	4	1	13.4 (0.5)
LF	2.58	10^{-4}	3	2	18.5 (0.6)
GF	2.58	2×10^{-4}	3	2	17.4 (0.9)
GLF	2.58	10^{-4}	3	2	18.2 (0.6)

[a] Stoichiometry and stability constants of oligopeptide–terbium complexes in methanolic solutions.

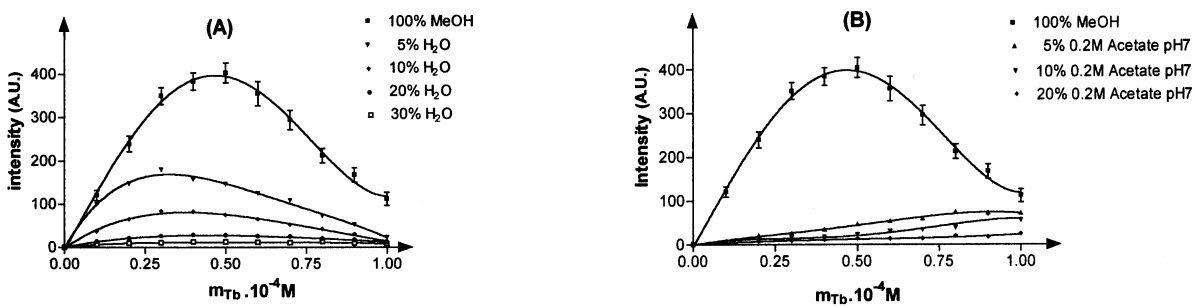

Fig. 7. Job's curve in the case of LF with TRF analysis ($\lambda_{ex} = 258$ nm, $\lambda_{em} = 545$ nm, td = 0.1 ms, tg = 5 ms). (A) In various hydromethanolic solutions without salt; (B) in various hydromethanolic solutions with 0.2 M sodium acetate salt.

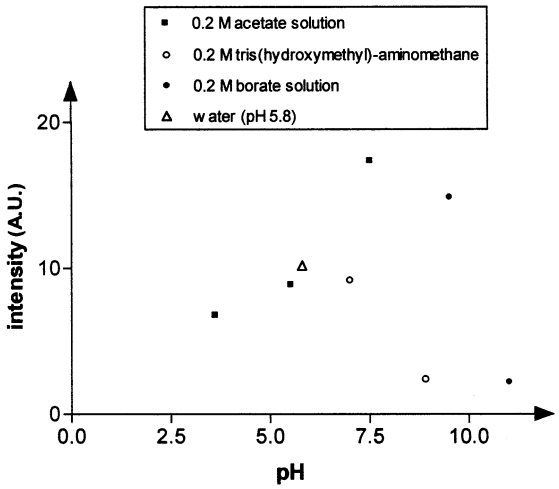

Fig. 8. Influence of dissolved salts and pH on emission at 545 nm ($\lambda_{ex} = 258$ nm) of a mixture of 6×10^{-5} M LF and 4×10^{-5} M $TbCl_3$ in hydromethanolic solution (60/40, v/v).

with a hard base such as oxygen rather than with a softer base such as nitrogen [16]. Thus, with respect to the pK_a of the free function COOH/COO^-, the stablest complexes are those with higher pK_a (Table 4). However, the steric conformation of the peptide with N-terminal phenylalanine is probably more favorable for access to the lanthanide environment, inducing binding with 4 moles of peptide.

To adapt these results for detection purposes after chromatographic separation of peptides, we studied the influence of pH on the TRF intensity of a mixture of 6×10^{-5} M LF and 4×10^{-5} M $TbCl_3$ in hydromethanolic solution (60/40, v/v). Fig. 8 shows that the best emission was obtained for the solution in 0.2 M pH 7 acetate/methanol 60/40 (v/v). Job's curves of LF were then performed in this hydromethanolic solution with and without dissolved acetate (used as a modifier in elution, and also proposed for reduction of water-quenching [25]). Fig. 7A confirms the well-known quenching of water on lanthanide fluorescence and indicates that the stoichiometric ratio of the complex has been modified, probably because of the formation of aquo complexes of oligopeptides [16]. Fig. 7B shows that acetate hindered the formation of complexes with the oligopeptides studied, and suggests that acetate terbium complexes occurred.

4. Conclusion

As theoretically expected, the formation of a complex between oligopeptide and terbium occurred, i.e. $(LF)_3(Tb)_2$, $(GF)_3(Tb)_2$, $(GLF)_3(Tb)_2$ and $(FL)_4Tb$, $(FG)_4Tb$. Complexes formed in methanolic solutions and showed high stability constants (p$K_d > 13$). Lanthanide chelate emission with a large Stokes shift (> 270 nm) seems promising for the detection of GLF and its derivatives as it allows the recording of emission spectra without interference from scattering phenomena. However, this preliminary study indicated some important limitations associated with lanthanide chelation, such as high methanolic content.

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