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SENSITIVE DETERMINATION OF PROTEINS BY ITS QUENCHING EFFECT ON FLUORESCENCE OF NEW TERBIUM(III) COMPLEX

INNA LEONENKO[1], DARIA ALEKSANDROVA[1], ALLA YEGOROVA[1]*, VALERY ANTONOVICH[1] and ALEKSANDR KARASYOV[2]

[1]A.V. Bogatsky Physico-Chemical Institute of National Academy of Sciences of Ukraine, Odessa 65080, Ukraine
[2]Active Motif Chromeon GmbH, Tegernheim 93105, Germany

Abstract: It is found that in hexamethylenetetramine (HMTA-HCl) buffer pH = 7.8, proteins can quench the fluorescence intensity of new terbium(III) complex with 6-[(1-hydroxy-3-oxo-6,7-dihydro-3H,5H-pyrido[3,2,1-ij]quinoline-2-carbonyl)-amino]-hexanoic acid (L). Based on this, a sensitive fluorimetric method for the determination of proteins is proposed. Under optimum conditions, the I_0/I is in proportion to the concentration of protein in the range of 0.1–40.0 μg/mL for bovine serum albumin (BSA), 0.1–70.0 μg/mL for human serum albumin (HSA) and 0.1–40.0 μg/mL for immunoglobulin G (IgG). Their detection limits (S/N = 3) are 0.03 μg/mL. The interaction mechanism for the luminescence quenching is also studied.

Keywords: fluorescence quenching; terbium; proteins

It is well known that the proteins are fundamental elements of life. So quantitative analysis of proteins is very important in the fields of chemical, biochemical, food and clinic analysis.

The traditional and widely used spectrophotometry methods for the determination of proteins are: the Lowry (1), Bradford (2), Coomassie bromophenol blue (3), bromocresol green (4), indigo carmine (5) and quercetin (6). These methods have limited sensitivity, narrow linear range or slow reaction time.

Therefore, many fluorometric methods are used for the assay of proteins because of their sensitivity, stability, simplicity and other advantages (7–9). Among these probes, rare earth chelate probes (10–17) are widely applied because they have the luminescence characteristics of rare earth ions, such as narrow emission bands, long luminescence decay times, large Stoke's shift and also have higher sensitivity. Therefore, there is great interest in search for novel ligands.

Here, we employ the new probe Tb(III)-L which is simple to prepare, water soluble, photostable in solution of physiological pHs and does not require the addition of luminescence enhancers such as organic solvents, surfactants and donor active additives.

The fluorescence intensity of terbium(III) complex is quenched considerably by proteins. Based on this phenomenon, a novel method for the determination of proteins has been developed.

EXPERIMENTAL

Materials

Proteins (BSA, HSA and IgG) were purchased from Sigma without further purification. Their stock solutions (1.0 μg/mL) were prepared by dissolving commercial proteins in doubly distilled water and stored at 0–4°C. The working standard solution of proteins was freshly obtained by dilution with the same solvent to final concentration of 100 μg/mL.

Stock solution (0.01 M) of terbium(III) chloride was prepared by dissolving the corresponding terbium oxide (Sigma, 99.99%) in hydrochloric acid. An excess of HCl was evaporated to a wet residue and diluted with water. The metal concentration was determined by complexometric titration with Arsenazo I as the indicator.

The ligand L was synthesized as described elsewhere (18). The stock solution of reagent (1×10^{-3} M) was prepared by dissolving accurate weights of the solid compounds in dimethylformamide (DMF).

* Corresponding author: e-mail: yegorova@interchem.com.ua

Working standard solutions were obtained by appropriate dilution of the stock solutions.

Hexamethylenetetramine (HMTA-HCl) buffer solution was prepared by dissolving HMTA (40.0 g) in 100 mL volumetric flask with water and adjusting the pH to 7.8 with HCl.

All of the used chemicals were of analytical grade or chemically pure; doubly-distilled water was used throughout.

Apparatus

All luminescence measurements (luminescence spectra, excitation spectra and lifetimes) were recorded using a Cary Eclipse luminescence spectrometer (Varian, Australia) equipped with a 150 W xenon lamp, using 1.0 cm quartz cells. The excitation wavelength was set at 300 nm and the fluorescence was measured using the peak height at 545 nm. All measurements were performed at room temperature (21–23°C). The pH values of solutions were measured using Lab 850 pH meter (Schott Instruments GmbH, Germany) with a glass electrode. All absorption spectra were recorded with an UV-2401 PC spectrophotometer (Shimadzu, Japan). Decay times were calculated from the obtained decay curves using Microcal Origin 6.0 software (1991–1999, Northampton, USA).

Procedures

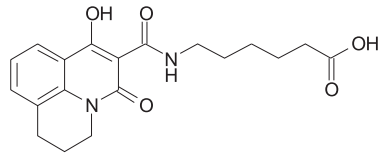
To 10 mL volumetric flasks solutions were added in the following order: 0.01; 0.05; 0.10; 0.20; 0.30; 0.40; 0.50; 0.60; 0.70; 0.80; 0.90; 1.00; 1.20; 1.30; 1.50; 1.80; 2.00; 2.50; 3.00; 3.50; 4.00; 5.00; 6.00; 6.50; 7.00 mL of working standard solution of proteins (BSA, HSA, IgG; standard human serum) (100 μg/mL); 1.0 mL of a working standard terbium chloride solution (1×10^{-5} M); 1.0 mL of L working standard solution (1×10^{-5} M); 0.4 mL of HMTA-HCl buffer. Simultaneously, a blank solution, which contained all the components with the exception of proteins, was prepared. The solutions were diluted with water up to 10 mL, stirred and stood for 5 min at room temperature. The luminescence intensity was measured in a 1 cm quartz cell at λ_{ex} / λ_{em} = 300 nm/545 nm. The quenched fluorescence intensity of Tb(III)-L by proteins was represented as I_0/I, where I_0 and I are the fluorescence intensities of the systems without and with protein, respectively. The standard curve method was used in the quantitative determination of trace amount of different proteins in the model sample.

For the assay of proteins in serum samples, the fresh samples must be diluted appropriately within the linear range of determination (1:1000). A portion (1.0 mL) of this sample solution was analyzed by the present method, using the standard calibration method.

RESULTS AND DISCUSSION

Spectral characteristics

The chemical structure of new ligand L – 6-[(1-hydroxy-3-oxo-6,7-dihydro-3H,5H-pyrido[3,2,1-ij]quinoline-2-carbonyl)-amino]-hexanoic acid is shown below:



The absorption spectra of L in aqueous solution display three bands with peaks with high molar absorption coefficient (ε) in the UV region: ε_{334} = 0.51×10^4 $M^{-1}\cdot cm^{-1}$; ε_{293} = 1.99×10^4 $M^{-1}\cdot cm^{-1}$; ε_{236} = 4.92×10^4 $M^{-1}\cdot cm^{-1}$. These coefficients give the possibility for effective absorption of light energy.

The energy of triplet level (T) of L (22,200 cm^{-1}) was calculated from phosphorescence spectra of its Gd(III) complex at 77 K. This energy is higher than the energy of level of the first excited Tb(III) ion state ($^5D_4$; 20,500 cm^{-1}), resulting in the possibility of energy transfer from ligand to lanthanide ions.

The excitation spectra of the complex Tb(III)-L monitored at 545 nm show an excitation maximum at 300 nm. Especially the 545 nm-band is hypersensitive to changes of the coordination environment of the respective complex. Therefore, the changes of the luminescence intensity of this band are most often used for analytical applications with Tb(III) complexes.

Luminescence is quenched strongly upon addition of different concentrations of proteins (BSA, HSA, IgG) (Fig. 1).

The lifetimes (τ) of the complexes Tb(III)-L and Tb(III)-L-proteins are constant (~680 μs) (Fig. 2). Static quenching between proteins and terbium(III) chelate can be explained by this fact.

Effect of pH and stoichiometry

The effect of pH on the fluorescence intensity of Tb(III)-L-BSA system was tested (Fig. 3). The complexation occurs in a wide range of pH values 5.5–10. The experimental results indicated that the fluorescence intensity was the strongest when pH was in the range of 7.5–8.5. Thus, pH = 7.8 (0.4 mL HMTA-HCl) was chosen for further research. Upon the lower pH values (in acid solutions) the degree of complexation is very low. In alkaline solutions (pH

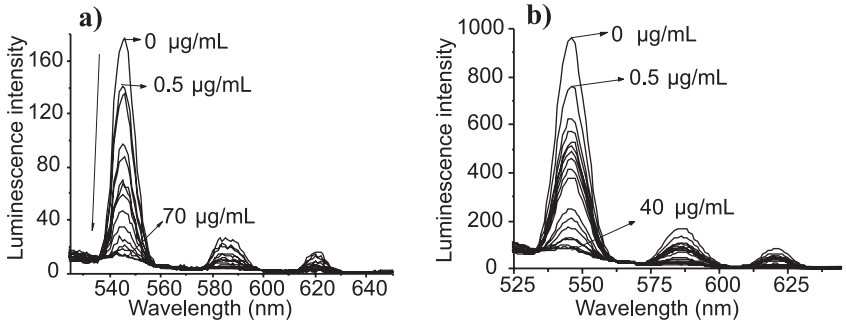


Figure 1. Luminescence spectra of Tb(III)-L complex in the presence of different concentrations of HSA (a), IgG (b) ($C_{Tb^{3+}} = C_L = 1\times10^{-6}$ M; $C_{protein}$ = 0.1 – 70.0 μg/mL)

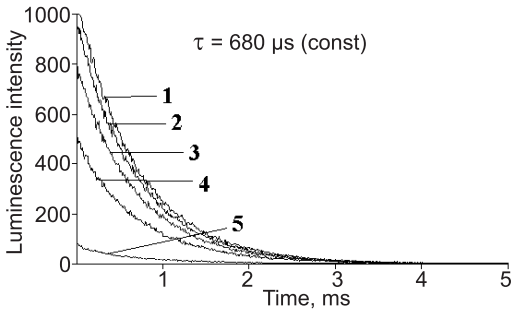


Figure 2. Decay curves for Tb-L in absence (1) and in presence of BSA (2 – 0.5 μg/mL, 3 – 1.0 μg/mL, 4 – 2.0 μg/mL, 5 – 5.0 μg/mL) ($C_{Tb^{3+}} = C_L = 1\times10^{-6}$ M; pH 7.8)

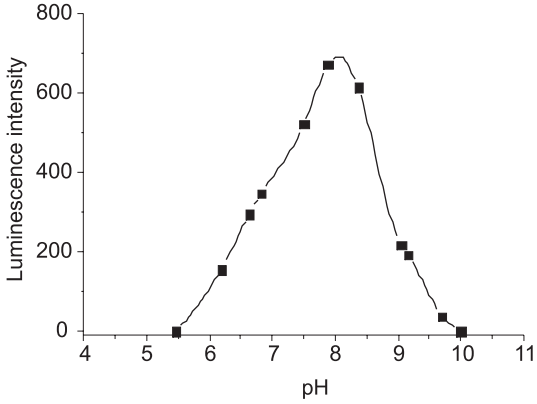


Figure 3. Effect of pH on the emission intensity of Tb-L complex in the presence of BSA ($C_{Tb^{3+}} = C_L = 1\times10^{-6}$ M; C_{BSA} = 5 μg/mL)

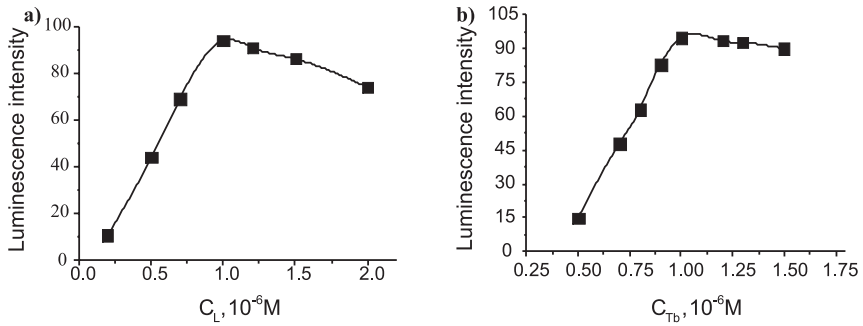


Figure 4. Effect of L (a) and Tb(III) (b) concentrations (C_{BSA} = 20 μg/mL; pH 7.8)

> 9), the decomposition of complex with the formation of the terbium hydroxide was observed.

The effect of the Tb^{3+} concentration on the luminescence intensity was investigated in the concentration range between 0.5–1.5 μM of Tb^{3+} with 5 μg/mL of BSA and with 1 μM of L at pH 7.8 (Fig. 4). The effect of the concentration of L on the luminescence intensity, in turn, was investigated at a constant Tb^{3+} concentration (1 μM). Optimal conditions were established at equal concentrations of

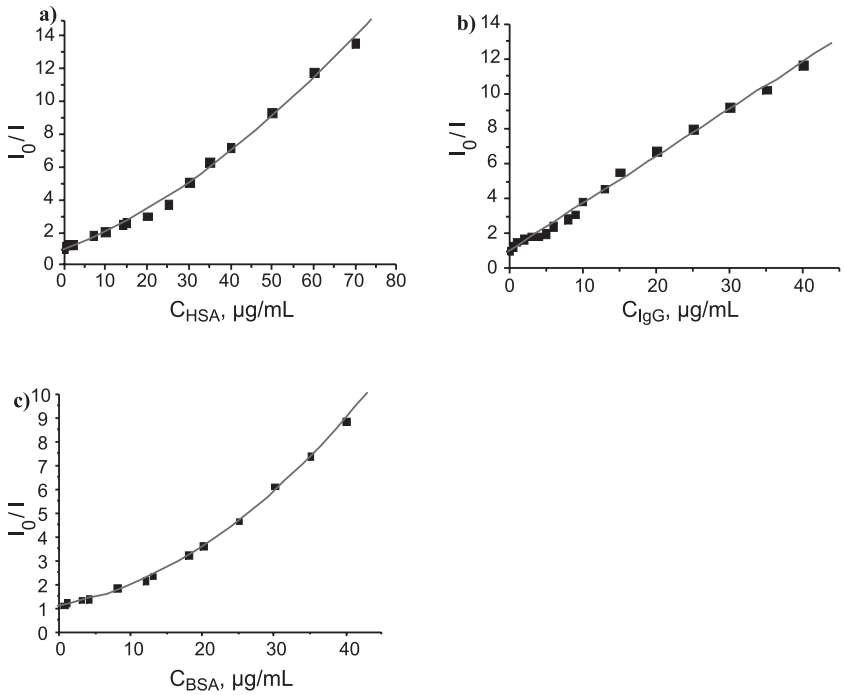


Figure 5. Stern-Volmer calibration plots for HSA (a), IgG (b) and BSA (c) determination ($C_{Tb^{3+}} = C_L = 1\times10^{-6}$ M; pH 7.8)

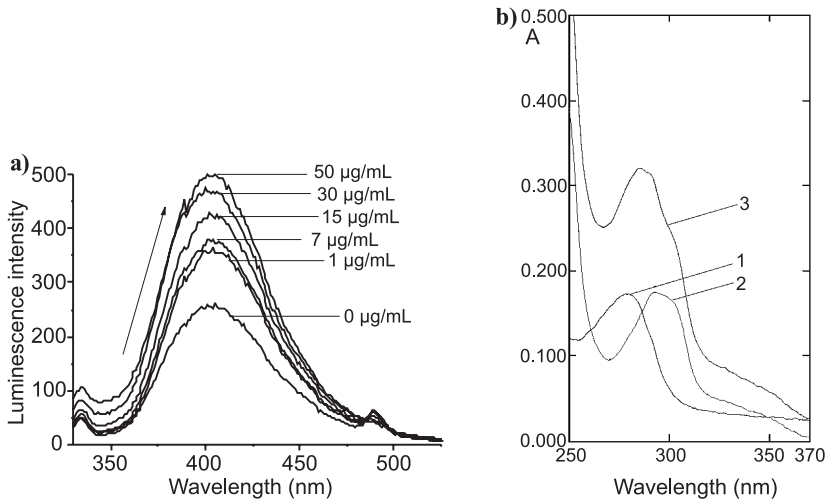


Figure 6. Luminescence spectra of L in complex Tb(III)-L in the presence of different concentration of HSA (a) (C_{Tb} C_L = × 10^{-6} M) and (b) absorbtion spectra: IgG (1); L(2); L in the presence of IgG (3) ($C_L = 1\times10^{-5}$ M; C_{IgG} = 100 μg/mL; pH 7.8)

Tb^{3+} and L (1 μM), which therefore were chosen for further experiments.

Effect of interfering substance

The interferences of the different substances, including common metal ions, anions and amino acids in the determination of 5 μg/mL of HSA were tested. As shown in Table 1, the most of amino acids and metal ions had no or little effect on the determination of HSA under the permission of ±5% relative error, but the Fe^{3+}, Cu^{2+}, phosphate ions and nucleic acid caused significant interference.

Table 1. Effect of interfering substances on fluorescence ($C_{Tb^{3+}} = C_L = 1$ µM; $C_{HSA} = 5$ µg/mL , pH = 7.8)

Interfering substances	Concentration [µM]	Change of I_0/I [%]
NaCl	50	–3.9
KCl	50	–3.8
$CaCl_2$	50	–2.7
NH_4NO_3	50	–4.5
$MgCl_2$	50	–3.8
$A1_2(SO_4)_3$	10	+2.8
NaH_2PO_4	10	–6.3
$Fe_2(SO_4)_3$	1	–4.6
$CuCl_2$	1	–4.4
Glucose	50	–4.5
Lactose	40	–3.3
L-Lysine	10	+0.8
L-Alanine	20	+1.4
L-Adenine	10	+2.8
L-Thymine	10	+2.5
L-Cytosine	10	+3.8
L-Guanine	10	+3.1
ctDNA	1	–5.1

Table 2. Analytical parameters for protein determination ($C_{Tb^{3+}} = C_L = 1\times10^{-6}$ M; pH 7.8).

Proteins	Linear range [µg/mL]	Regression equation ($c = C_{protein}$ [µg/mL])	Correlation coefficient	Detection limit [µg/mL]
BSA	0.1–40.0	$I_0/I = 1.12 + 0.054c + 0.004c^2$	0.9989	0.03
HSA	0.1–70.0	$I_0/I = 0.96 + 0.101c + 0.001c^2$	0.9952	0.03
IgG	0.1–40.0	$I_0/I = 1.01 + 0.271c$	0.9974	0.03

Table 3. Results of the determination of proteins in synthetic samples (n = 5). Conditions: $C_{Me^{n+}} = 1\times10^{-5}$ M; $C_{Glucose} = 1\times10^{-5}$ M; $C_{L\text{-}Alanine} = 5\times10^{-6}$ M.

Foreign substances	Protein	Added [µg/mL]	Found [µg/mL]	Recovery (%)	RSD [%]
Na^+, K^+, Ca^{2+}, Glucose, L-Alanine	BSA	0.50 5.00 20.00	0.51 ± 0.03 4.97 ± 0.23 19.84 ± 0.54	102.0 99.4 99.2	4.3 3.8 2.2
	HSA	0.50 5.00 20.00	0.49 ± 0.02 4.91 ± 0.24 20.05 ± 0.47	98.0 98.2 100.3	4.1 3.9 1.9
	IgG	0.50 5.00 20.00	0.51 ± 0.02 5.08 ± 0.22 20.03 ± 0.52	102.0 101.6 100.2	3.9 3.5 2.1

Analytical application

The calibration graphs and detection limits

Under the optimum conditions, the different concentrations of proteins were added to Tb(III)-L = 1:1 complex. The plots of Stern-Volmer were obtained (Fig. 5) and shown in Table 2. I_0 and I were measured at $\lambda_{exc} = 300$ nm and $\lambda_{em} = 545$ nm, where I_0 and I are the relative luminescence intensities of

Table 4. Results of the determination of proteins in serum samples (n = 5).

Sample	Methods	Found [mg/mL]	Average [mg/mL]	RSD [%]
Serum 1	Proposed method	73.2; 78.3; 76.8; 72.4; 78.6	75.9 ± 3.6	3.8
	UV method (1)	71.8; 76,2; 73.5; 74.8; 77.1	74.7 ± 2.6	2.8
Serum 2	Proposed method	68.4; 66.4; 70.1; 71.9; 66.7	68.7 ± 2.9	3.4
	UV method (1)	69.2; 67.5; 70.8; 68.5; 66.3	68.5 ± 2.1	2.5

Table 5. Comparison of sensitized luminescence methods for the determination of proteins

Fluorogenic reagent	Protein	Conditions	Linear range [μg/mL]	LOD [ng/mL]	Ref.
Eu^{3+}-doxycycline	HSA	pH = 10.2; NH_4OH-NH_4Cl	0–9.2 9.2–34.5	64.0 115.0	(10)
Eu^{3+}-tertacycline	HSA	pH = 10.2; NH_4OH-NH_4Cl	0–1650	165000	(11)
Eu^{3+}-chlortertacycline	HSA BSA	pH = 8.5; Tris-HCl	0.8–10.0 0.2–10	33 8.9	(12)
Eu^{3+} -thenoyltrifluoroacetone	HSA	pH = 5.3; Britton -Robinson buffer; sodium dodecyl benzene sulfonate	0–5.1 5.1–44.4	20.7	(13)
Tb^{3+}-enoxacine	HSA	pH = 7.8; Tris-HCl	0.12–13	65.5	(14)
Tb^{3+}- thenoyltrifluoroacetone	BSA HSA Egg albumin (EA)	pH = 8.0; HMTA-HCl sodium dodecyl benzene sulfonate	0.004–7.5 0.005–15.0 0.01–7.5	0.5 0.8 2.0	(15)
Tb^{3+}-benzoylacetone	BSA HAS EA	pH = 8.2; Tris-HCl sodium dodecyl benzene sulfonate	0.010–6.0 0.010–6.0 0.03–6.0	3.9 4.0 8.5	(16)
Tb^{3+}-oxolinic acid	BSA HSA EA	pH = 6.0; NH_4Ac-HCl, sodium dodecyl sulfate	0.05–10.0 0.1–10.0 0.4–10.0	21.0 25.0 50.0	(17)
Tb^{3+}-L	BSA HSA IgG	pH = 7.8; HMTA-HCl	0.1–40.0 0.1–70.0 0.1–40.0	31.0 30.0 33.0	this work

the system without and with proteins, respectively, C is the concentration of protein (μg/mL). As can be seen from Figure 5a,c, the Stern-Volmer plot had been found to be non-linear with an upward curvature and obeyed the polynomial equation for BSA and HSA. This results suggest that both static and dynamic quenching processes are responsible for the observed positive deviation in the Stern-Volmer plot. As can be seen from Figure 5b, the Stern-Volmer plot had been found to be linear for IgG. This result suggests that static quenching process dominates. The signal-to-noise ratio of 3 was considered as the limit of detection (LOD). The LODs for proteins were found and showed in Table 2.

Accuracy and precision of the analysis was evaluated by carrying out the recovery study at three different concentrations. The results of recovery study indicate that the proposed method is accurate for estimation of proteins (Table 3).

Determination of proteins in serum samples

The developed method was applied to the determination of proteins in the human serum and compared with the traditional UV spectrophotometric method (1). In order to determine the total proteins in human serum samples, it is necessary to construct the calibration curve of standard human serum because the calibration curves of HSA and human

globulin have different slopes. The construction of calibration curve and the determination of serum samples were then performed according to the procedures described above.

The results are shown in Table 4. It can be seen that the accuracy and precision of the method are satisfactory and that the determined protein contents in the sample were in good agreement with data obtained by the other method. This indicates that this method is suitable for practical application. By comparison with some methods, as shown in Table 5, the present method has high sensitivity and selectivity, good stability and wide linear response range for determining the protein concentration.

Interaction mechanism of the system

Based on the above facts, it is considered that Tb-L complex has at least two different modes of interaction with protein which lead to significant decrease of luminescence intensity.

Our research indicated that Tb^{3+} could combine with L to form 1:1 complex with positive charge, which can emit strong characteristic fluorescence of Tb^{3+}. Under the experimental condition (pH 7.8) BSA is negatively charged and can react with Tb^{3+}-L complex through electrostatic force, which resulting in the fluorescence quench of Tb^{3+}-L system (Fig. 1) and increase of own luminescence of L (Fig. 6a). It is possible to explain the given effect by back energy transfer from excited state of Tb^{3+} to L, that is connected with a small gap between the triplet energy of the sensitizer (22,200 cm^{-1}) and the excited state of Tb^{3+} (20,500 cm^{-1}).

On the other hand, the ligand has a functional group (carboxyl group), which makes its able to bind to amino group of proteins. That is confirmed by changing of absorption spectra of free ligand and BSA as compared to spectrum of L-BSA (Fig. 6b). As a result, the energy loss of Tb^{3+}-L complex is observed, which lead to the fluorescence quench.

CONCLUSIONS

In this paper, we described that the fluorescence intensity of the new terbium complex Tb^{3+}- L remarkably decreased with the addition of proteins. Based on this, a new fluorimetric method for the determination of proteins had been reported. Under optimum conditions, the quenched intensity of fluorescence was in proportion to the concentration of proteins in the wide range. The developed method has been applied to the determination of proteins in serum samples. The interaction mechanism was also studied.

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