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NOVEL LUMINESCENT PROBE BASED ON A TERBIUM(III) COMPLEX FOR HEMOGLOBIN DETERMINATION

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We have studied the spectral luminescent properties of Tb(III) and Eu(III) complexes with a number of novel derivatives of oxoquinoline-3-carboxylic acid amides (L_1–L_5). We have observed quenching of the luminescence of 1:1 Tb(III)–L_{1-5} complexes by hemoglobin (Hb), which is explained by resonance energy transfer of electronic excitation from the donor (Tb(III)–L_{1-5}) to the acceptor (Hb). Using the novel luminescent probe Tb(III)–L_1, we have developed a method for determining Hb in human blood. The calibration Stern–Volmer plot is linear in the Hb concentration range 0.6–36.0 μg/mL, detection limit 0.2 μg/mL ($3\cdot10^{-9}$ mol/L).

Keywords: *hemoglobin, terbium complexes, luminescence.*

Introduction. Hemoglobin (Hb) is a complex iron-containing protein in animal and human erythrocytes that can reversibly bind oxygen [1] and enables transport of oxygen to the tissues. Hemoglobin consists of a protein part (globin) and an iron-containing part (heme). The molecular weight of human hemoglobin is assumed to be 68,800. Determination of hemoglobin content in human blood is one of the most important and widely used tests. For routine laboratory studies, photometric methods are preferred (the hemiglobin cyanide method, the hemichrome method, and the hemiglobin azide method), the uncertainty in which is ≤2% and the lower detection limit is ~25 g/L ($3.6\cdot10^{-4}$ mol/L) [2].

Although the metrological characteristics of the photometric methods meet the requirements for large-scale blood tests, certainly the sensitivity of Hb determination needs to be improved. Fluorescent methods are promising in this regard. When hemoglobin is exposed to UV and visible light, intrinsic luminescence appears, which cannot be used for analytical purposes because of its low excitation efficiency. Today, a timely problem for fluorescent analysis is to increase the sensitivity of hemoglobin determination using luminescent probes, the emission of which changes significantly in the presence of hemoglobin (the emission increases or is quenched) [3–8].

Some capabilities of the familiar methods for high-sensitivity fluorimetric determination of Hb in human biological fluids are systematically organized in Table 1. Generally the analyte is determined indirectly from the emission of the products of various reactions, in most of which the hemoglobin plays the role of catalyst. In this case, the specifications for the analytical procedures must be strictly followed. Although the proposed methods are mainly considered highly selective, we should take into account the interfering effect of comparable amounts of some *d*-metal ions [4–6] and bovine serum albumin [4, 5]. A flow-injection chemiluminescence method for Hb determination in blood samples has also been developed, based on the ability of hemoglobin to enhance luminescence in the H_2O_2–$K_4Fe(CN)_6$–fluorescein system in the presence of CdTe quantum dots [9].

Lanthanide complexes are widely used to determine biologically active substances (proteins, enzymes, nucleic acids, drugs, etc.) [10–13]. The main requirements for such lanthanide complexes to be used as probes in bioanalysis is high quantum yield and high kinetic stability, and also good solubility in water at optimal physiological pH values. In a number of cases, it is possible to not only determine biologically active substances whose components form complexes with lanthanide ions and sensitize their 4*f*-luminescence, but also to use luminescent probes utilizing the quenching of their emission by the biologically active substances, without chemical reaction with the central ion [14].

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TABLE 1. Characteristics of Luminescent Methods for Hemoglobin Determination

Reagent	Interval of linearity, mol/L	Detection limit, mol/L	Interfering substances (mole ratios or concentrations)	Literature reference
p-Cresol	$1\cdot10^{-9}$–$8\cdot10^{-8}$	$3.4\cdot10^{-10}$	Fe(III) (20), Cu(II) (10)	[2]
Rhodamine B	$2\cdot10^{-10}$–$1.2\cdot10^{-8}$	$8.6\cdot10^{-11}$	Fe(II) (20), ascorbic acid (20), BSA (10 μg/mL)	[3]
Al(III) complex with tetraaminophthalocyanine	$5\cdot10^{-11}$–$1.2\cdot10^{-8}$	$1.5\cdot10^{-11}$	Fe(III) (100), Cu(II) (10), BSA (5 μg/mL	[4]
Thiamine	$2\cdot10^{-10}$–$1\cdot10^{-7}$	$4.6\cdot10^{-11}$	Fe(III) (100), Cu(II) (10), Co (10), Mn (10)	[5]
bis-Salicylaldehyde–*o*-phenylenediimide	$5\cdot10^{-10}$–$5\cdot10^{-8}$	$2.6\cdot10^{-11}$	Ni(II) (20), NH_4^+ (10)	[6]
Fluorescein–5-isothiocyanate–DNA	$5\cdot10^{-9}$–$2\cdot10^{-7}$	$2\cdot10^{-9}$	BSA (6.4 μg/mL)	[7]
H_2O_2–$K_4Fe(CN)_6$– fluorescein in the presence of CdTe	$7.35\cdot10^{-9}$–$2.5\cdot10^{-6}$	$1.8\cdot10^{-9}$	Ca (II) (100), ascorbic acid (50), BSA (50), Zn (II) (25), Fe (III) (10)	[8]

The aim of this work was to develop a simple and sensitive method for quantitative determination of hemoglobin from the quenching of the luminescence of terbium(III) complexes containing a novel efficient sensitizer.

The Experiment. We used at least analytical grade reagents and twice-distilled water. Standard solutions of terbium chloride (0.1 mol/L) were prepared by dissolving the corresponding high-purity oxide (which was precalcined in a muffle furnace for 1 h at 650–700°C) in hydrochloric acid (1:1), followed by evaporation of the excess on a water bath. The dry residue was dissolved in distilled water and diluted up to the required volume. The concentration of the solution obtained was monitored complexometrically with the indicator arsenazo I in urotropin buffer solution at pH 7.0 ± 0.2.

The reagents (the sensitizers L_1-L_5) were synthesized, identified, and purified at the National Pharmaceutical University (Kharkov, Ukraine) [15]. Solutions ($1\cdot10^{-3}$ mol/L) of the reagents were obtained by dissolving exactly weighed amounts in dimethylformamide (DMF). Hb solution (1.2 mg/mL): 0.5 mL of GK-2 Hb solution (110–130 g/L, Agat-Med OOO, lot 340/580111) were placed in a 50 mL volumetric flask and brought up to the mark with water. Hb solution (120 μg/mL): 5.0 mL of Hb solution (1.2 mg/mL) were placed in a 50 mL volumetric flask and brought up to the mark with water. The acidity of the solutions was established using a 2.86 mol/L (40%) aqueous urotropin solution (pH 7.5).

The luminescence and luminescence excitation spectra and also the luminescence decay curves were recorded on a Cary Eclipse spectrofluorimeter (Varian, Australia) with a 150 W xenon lamp; the absorption spectra were recorded on a UV-2401 PC spectrophotometer (Shimadzu, Japan). The pH values of the solutions were measured using a Lab 850 pH meter (Schott Instruments GmbH, Germany) with a glass electrode, calibrated using standard buffer solutions. All the spectral measurements were made at room temperature.

Results and Discussion. The novel organic reagents (sensitizers of 4*f* luminescence), the pyridine-substituted oxoquinoline-3-carboxylic acid amides L_1–L_5:

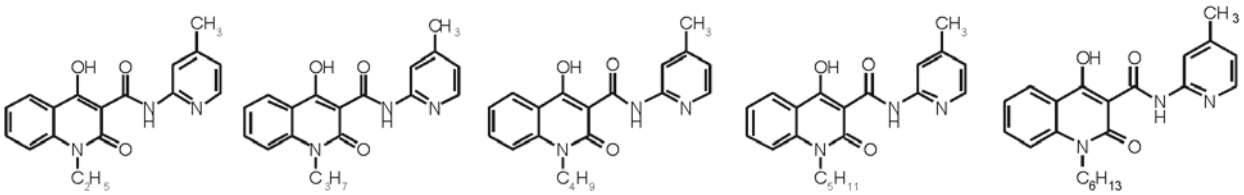

TABLE 2. Spectral Luminescent Characteristics of the Reagents (L_1–L_5) and Their Complexes (1:3) with Lanthanide Ions

Reagent	T^*, cm^{-1}	λ^*, nm	$\varepsilon^* \cdot 10^4$	λ_{ex}, nm	Tb(III)		Eu(III)	
					Φ	τ, μs	Φ	τ, μs
L_1	22,220	313	2.49	335	0.48	1350	0.32	1015
L_2	22,370	313	2.53	336	0.47	1240	0.40	1020
L_3	22,150	313	2.46	334	0.47	1130	0.34	930
L_4	22,120	313	2.43	326	0.45	1080	0.28	860
L_5	22,200	313	2.45	327	0.40	1090	0.30	830

Note. $C_{Tb} = C_{Eu} = 1 \cdot 10^{-5}$ mol/L; $C_{L*} = 5 \cdot 10^{-5}$ mol/L; pH 7.5.

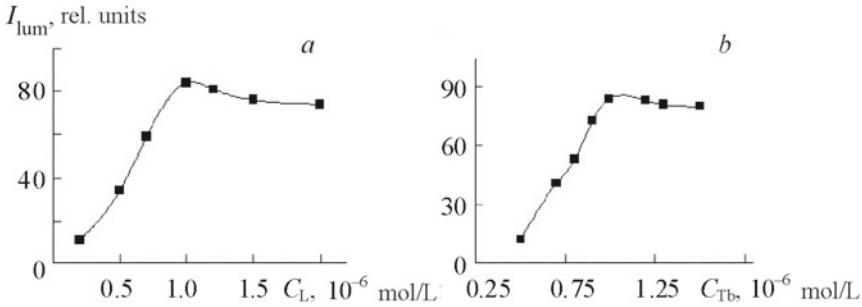

Fig. 1. I_{lum} of the system Tb(III)–L_1–Hb vs. the concentrations of L_1 (a) and Tb(III) (b); C_{Hb} = 20 μg/mL.

were synthesized according to predictions obtained based on chemometrics [16, 17]. The reagents L_1–L_5 are characterized by the presence of absorption bands with high molar extinction coefficients in the UV region of the spectrum, which is responsible for the efficient absorption of excitation energy. Triplet levels of the ligands, calculated from the phosphorescence spectra of their Gd(III) complexes at 77 K, are found at 22,220–22,370 cm^{-1} and exceed the energy of the level for the first excited state of the Tb^{3+} ion (20,500 cm^{-1}) and the Eu^{3+} ion (17,300 cm^{-1}), which determines the possibility of intramolecular transfer of the energy absorbed by the ligand to the lanthanide ion levels and leads to a significant increase in their luminescence. From the spectral luminescent characteristics presented in Table 2 for complexes of L_1–L_5 with Tb(III) and Eu(III) ions, it follows that the complex Tb(III)–L_1 has the highest quantum yield, and this complex was used as the luminescent probe for Hb determination.

Interaction of Hb with the luminescent probe Tb(III)–L_1 is observed over a broad acidity range (pH 4.5–10.0); the maximum increase in luminescence intensity is observed at pH 7.5–8.5, which is achieved by adding a 40% urotropin solution (pH 8.0). For constant Hb concentration (20 μg/mL), we studied the dependences of I_{lum} on the concentration of Tb(III) and L_1 for the system Tb(III)–L_1–Hb. We established that the optimal concentrations of the components are $C_{Tb} = C_{L_1} = 1 \cdot 10^{-6}$ mol/L (Fig. 1).

As we see from Fig. 2a, dramatic quenching of the luminescence of this complex occurs when different Hb concentrations are added, and the luminescence lifetime of the complex Tb(III)–L_1 varies considerably for different Hb concentrations (Fig. 2b). Quenching of the 4*f* luminescence of the complex Tb(III)–L_1 by hemoglobin can be explained by energy transfer from the complex Tb(III)–L_1 (the donor) to Hb (the acceptor). Evidence in favor of resonance transfer of the electronic excitation energy (FRET, Förster resonance energy transfer) in this case comes from the overlap between the absorption spectrum of hemoglobin and the luminescence spectrum of the complex Tb(III)–L_1 (Fig. 3). The Förster radius (R_0 = 47 Å) and the overlap integral ($\int_{over} = 2.9 \cdot 10^{13}$ $M^{-1} \cdot cm^{-1} \cdot nm^4$) for overlap between the luminescence spectrum of Tb(III)–L_1 and the absorption spectrum of Hb (Fig. 3) are calculated in the SetaFret program, taking into account the distribution of the emission quantum intensity in the luminescence spectrum of the donor and the molar absorption coefficient of the acceptor.

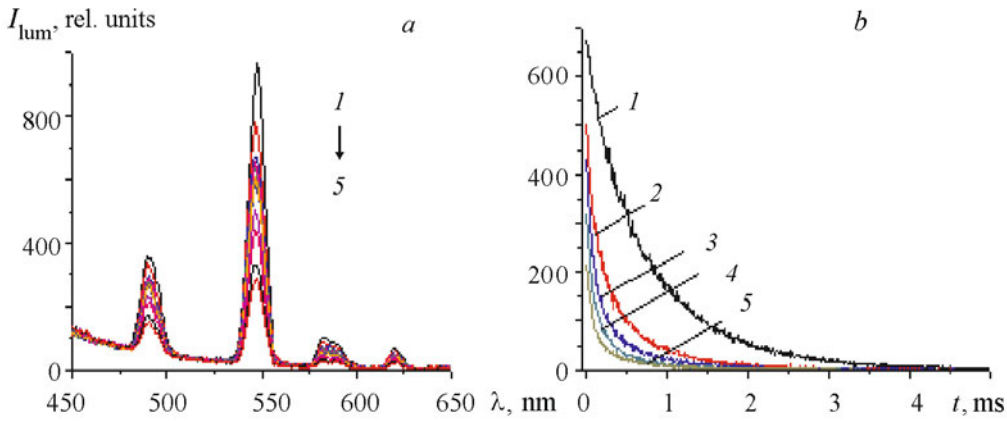

Fig. 2. Luminescence spectra (a) and luminescence decay curves (b) for the complex Tb(III)–L_1 for C_{Hb} = 0 (1), 2.4 (2), 7.2 (3), 18 (4), and 36 (5) μg/mL; t = 734 (1), 342 (2), 220 (3), 180 (4), and 165 (5) μs; $C_{Tb} = C_{L_1} = 1 \cdot 10^{-6}$ mol/L.

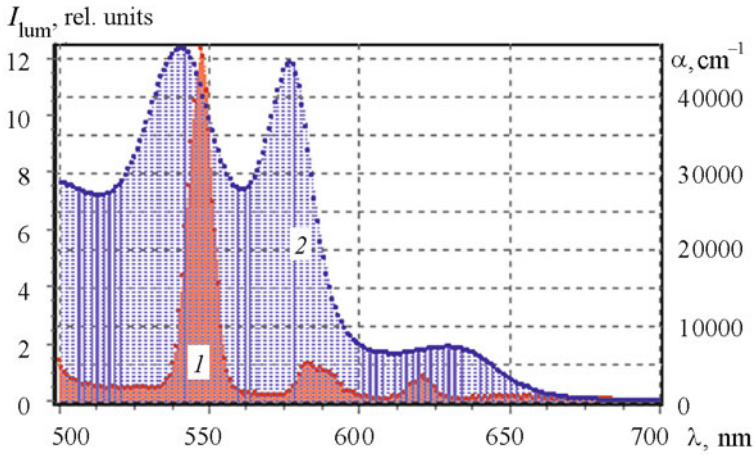

Fig. 3. Overlap of the luminescence spectrum of the complex Tb(III)–L_1 (1) with the absorption spectrum of hemoglobin (2).

The dependence of I_{lum} of the complex Tb(III)–L_1 on the Hb concentration and the calibration Stern–Volmer plot are presented in Fig. 4. The calibration Stern–Volmer plot is linear in the Hb concentration range 0.6–36.0 μg/mL and the detection limit is 0.2 μg/mL ($3 \cdot 10^{-9}$ mol/L). The efficiency of quenching of the luminescence of Tb(III) in the L_1 complex is proportional to the Hb concentration according to the Stern–Volmer law:

$$I_0/I - 1 = K_{SV} C_{Hb},$$

where I_0 and I are the luminescence intensities for Tb(III) without the quencher and in the presence of the quencher; C_{Hb} is the concentration of the quencher; K_{SV} is the Stern–Volmer constant.

We determined the quenching constant for the donor (Tb(III)–L_1) in the presence of Hb: $K_{SV} = (4.12 \pm 0.17) \cdot 10^6$ L/mol. The values we obtained for the quenching constant for quenching of the terbium ions in the L_1 complex by hemoglobin, and also the overlap integral and the Förster radius allow us to characterize Hb as an efficient quencher of 4*f* luminescence for Tb(III) ions. We must note the selectivity of Hb determination using the luminescent probe Tb(III)–L_1. It has been experimentally established that Fe(II, III) ions ($5 \cdot 10^{-8}$ to 10^{-6} mol/L) and albumins (50–100 μg/mL) do not cause quenching of the luminescence in the Tb(III)–L_1 complex.

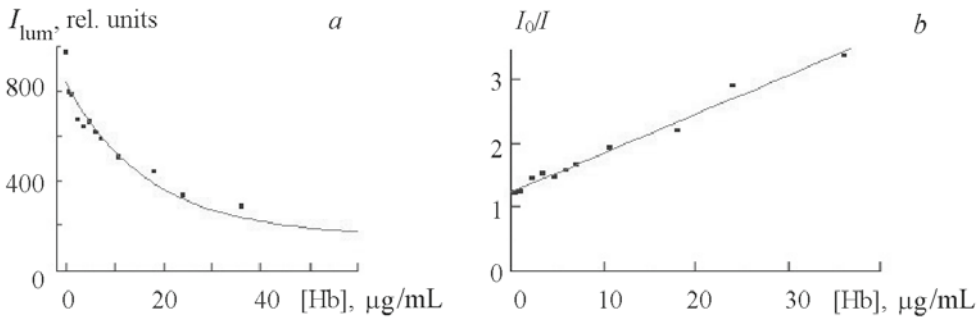

Fig. 4. Luminescence intensity for the complex Tb(III)–L_1 vs. the Hb concentration (a) and the calibration Stern–Volmer plot (b); $C_{Tb} = C_{L_1} = 1 \cdot 10^{-6}$ mol/L.

TABLE 3. Results of Determination of the Hemoglobin Concentration in Blood Samples (n = 5, P = 0.95) by Luminescent and Hemoglobin Cyanide Methods

Sample	$[Hb]_{lum}$, g/L	S_r	$[Hb]_{hem}$, g/L	S_r
1	145 ± 8	0.046	139 ± 7	0.041
2	122 ± 6	0.037	126 ± 5	0.032

Hemoglobin Determination in Blood Samples. ***Calibration curve.*** 0.05, 0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.90, 1.50, 2.00, 3.00 mL of the working Hb solution (120 μg/mL) were each added to 10 mL volumetric flasks (five parallel measurements). 1.0 mL of a $1 \cdot 10^{-5}$ mol/L solution of terbium chloride, 1.0 mL of a $1 \cdot 10^{-5}$ solution of L_1, and 0.4 mL of a 40% urotropin solution were added to each flask. The volumes were brought up to 10.0 mL with water and the mixture was stirred. In parallel, we prepared a solution of the control, which contains all the components except the protein. After 5 minutes, we measured the luminescence intensity at λ_{em} = 545 nm (λ_{ex} = 317 nm) at each point (I) and the luminescence intensity for the control (I_0).

Working solution of blood. 0.5 mL of the blood sample was placed in a 50.0 mL volumetric flask, the volume of the solution was brought up to the mark with water, and the mixture was stirred; 5.0 mL of the solution obtained was placed in a 50.0 mL volumetric flask, the volume of the solution was brought up to the mark with water, and the mixture was stirred.

2.0 mL each of the working solution of blood (five parallel measurements) were added to 10 mL volumetric flasks. 0.1 mL of a $1 \cdot 10^{-4}$ mol/L terbium chloride solution, 0.1 mL of a $1 \cdot 10^{-4}$ mol/L solution of L_1, 0.4 mL of the 40% urotropin solution were added to each flask. In parallel, we prepared a solution of the control, which contains all the components except for the working solution of blood. The solutions were brought up to 10.0 mL with water and stirred. After 5 minutes, we measured I_{lum} at λ_{em} = 545 nm (λ_{ex} = 317 nm). The Hb concentration in the blood samples was established using the calibration curve (taking dilution into account).

The correctness of the luminescent method was tested by comparing the results of the Hb determination in the blood samples obtained with data established in the clinical laboratory of Odessa State Medical University by the photocolorimetric hemiglobin cyanide method [18] (Table 3). The agreement between the results obtained by the two different methods is satisfactory.

Conclusions. We have demonstrated the feasibility of using a terbium(III) complex with the derivative 1-ethyl-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxylic acid (4-methylpyridine-2-yl)amide as a novel luminescent probe for sensitive determination of hemoglobin in blood samples. The proposed method is selective relative to Fe(II,III) ions and albumins, has satisfactory metrological characteristics, and is simple to perform.

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