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ORIGINAL PAPER

Enhancement of Europium Emission Band of Europium Tetracycline Complex in the Presence of Cholesterol

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Abstract We report here the observation, for the first time, of the enhancement of Europium-Tetracycline complex emission in cholesterol solutions. This enhancement was initially observed with the addition of the enzyme cholesterol oxidase, which produces H_2O_2, the agent driver of the Europium tetracycline complex, to the solution. However, it was found that the enzyme is not needed to enhance the luminescence. A calibration curve was determined, resulting in a simple method to measure the cholesterol quantity in a solution. This method shows that the complex can be used as a sensor to determine cholesterol in biological systems.

Keywords Cholesterol · Europium · Fluorescence · Optical Spectroscopy

Introduction

When in aqueous solution, lanthanide ions exhibits weak luminescence due to its small absorption cross section and strong energy transfer to surrounding water molecules [1, 2]. However, when the lanthanide is bonded to certain types of ligands, the ion luminescence can be increased. This increase is due to the ligand large absorption and an antenna-effect [3] that transfers the absorbed energy to the lanthanide through an intramolecular process [4], whose efficiency depends on the chemical nature of the ligand. The ion luminescence is also enhanced by the isolation that the ligand provides from the water molecules, preventing energy transfer to them. One such ligand is the tetracycline [5], an antibiotic that has an absorption band around 365 nm, and is also a chelating molecule, partially isolating the lanthanide from the water molecules. When combined with europium trivalent ions, the complex formed (europium-tetracycline–EuTc) has an absorption band centered around 400 nm, presenting a large Stokes-Shift (approximately 210 nm) due to an efficient antenna-effect, strongly emitting around 615 nm [6], in the red region of the visible spectrum. This luminescence was observed to increase up to 15 times in the presence of H_2O_2 due to these molecules capability of displacing water molecules from the Eu neighborhood [7]. These characteristics make the complex highly sensitive and specific for H_2O_2 detection [8]. As an additional advantage, the EuTc complex can be excited by commercial LEDs and lasers, and works in neutral pH [9].

The cholesterol is a steroid alcohol with chemical formula $C_{27}H_{45}OH$, that derives its name from the Greek roots *chol-* (bile) and *stereos* (solid), and was first identified in solid form in gallstones in 1784 [10]. The hydroxyl group is the only polar part of the molecule, resulting in a small region that is water-soluble in a fat-soluble molecule, configuring an amphipathic molecule. Cholesterol is required to build and maintain cell membranes of all body tissues, and is transported in the blood plasma of all animals. It aids in the manufacture of bile (which helps to digest fats), and is also important for the metabolism of fat soluble vitamins such as vitamins A, D, E and K. It is the major precursor for the synthesis of vitamin D, fundamental

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component [11] of various steroid hormones, including cortisol and aldosterone, and of the sex hormones progesterone, estrogen, and testosterone. Most cholesterol is not dietary in origin, being synthesized internally. Cholesterol is present in higher concentrations in tissues which either produce more or have more densely-packed membranes, as the liver, spinal cord and brain, and also in atheromas.

Low-density lipoproteins (LDL) are the main carriers of cholesterol, playing a critical role in the human cholesterol metabolism. The structure of the LDL particles have two well-defined regions, a core and a surface layer [12]

The excess of cholesterol associated with LDL in the blood is one of the main risk factors for the development of cardiovascular diseases, including atherosclerosis and hypertension, among others [13, 14]. According to the American Heart Association, normal levels of the sanguineous total cholesterol are below of 200 mg/dl, while concentrations above of 240 mg/dl are high risk factors for coronaries illnesses [15]. For these reasons, cholesterol has become one of the main parameters to be determined in routine clinical diagnosis.

Several schemes have been proposed in the literature for determining cholesterol concentrations. Enzymatic methods have practically replaced chemical methods based on the classical Liebermann-Burchard reaction, used traditionally for free and total cholesterol determination, since enzymes ensure the specificity and selectivity required for these kinds of assays. The current enzymatic methods are based on cholesterol esterase (ChE) and Cholesterol Oxidase (ChOx). Oxygen consumption or the production of hydrogen peroxide during the enzymatic reaction can be measured using either an electrochemical method [16] or an optical one, such as spectrophotometry [17] or chemiluminescence [18]. In some cases the enzymatic reaction has been coupled with fiber-optic technology [19, 20]. In this paper we describe two new optical methods for total cholesterol determination, both based on the enhancement of the EuTc complex emission. The first method is based in the hydrogen peroxide production by an enzymatic reaction, and the other one is a direct sensor method of clinical analysis for the determination of cholesterol in solution.

Materials and methods

The EuTc complex was prepared starting from inorganic salts with analytical purity, obtained from Sigma Aldrich and Molecular Probe. All solutions were prepared in 10 mmol l^{-1} 3-(N-Morpholino) propanesulfonic acid (Mops, from Carl Roth) buffer with pH 6.9. The tetracycline-HCl solution used is a secondary pattern gently provided by Bunker Indústria Farmacêutica Ltda. Cholesterol solution (CAT No. 01401; 200 mg/dl) used in this work was obtained from Laborlab Produtos para Laboratórios LTDA. The prepared solutions were:

Solution I	Mops buffer—544.1 mg of Mops salt in 200 ml of distilled water (pH=6.9)
Solution II	63 μmol l^{-1} solution of Eu^{3+} – 2.3 mg of $EuCl_3 \cdot 6H_2O$ in 10 ml solution I
Solution III	21 μmol l^{-1} solution of tetracycline–1.0 mg of tetracycline in 10 ml of solution I
Solution IV	Solution IV EuTc solution–Mix 10 ml of solution II and 10 ml of solution III.
Solution V	Solution V EuTc-cholesterol solutions–Mix of 1 ml of solution IV with x μl (x=10, 20, 30, 40, 50, 60, 70, 80, 90, 100) of cholesterol solution (200 mg/dl).

The absorption spectra of all samples were measured in the range 200–500 nm at room temperature using a Varian Cary 17D Spectrometer. The emission spectra were obtained by exciting the samples, inside a 1 mm optical path cuvette, with a 150 W Xenon lamp. The emissions of the samples were analyzed with a 0.5 m Spex monochromator and a PMT detector. The signal was amplified with an EG&G 7220 lock-in and processed by a computer. Time resolved luminescence spectroscopy was obtained from a Cary Eclipse Fluorescence spectrophotometer. The relative errors in the emission and lifetime measurements are estimated to be under 5%.

Results

The optical properties of the EuTc were investigated for the solutions with (solution V) and without (solution IV) pure cholesterol in their compositions.

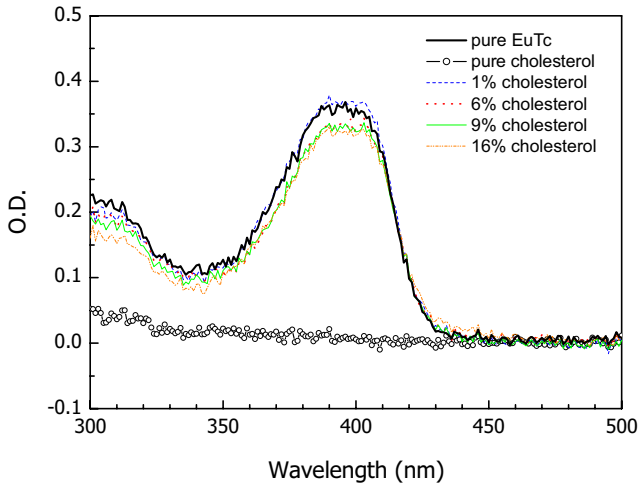

Fig. 1 Absorption spectra for cholesterol, EuTc and EuTc + cholesterol solutions. The absorption spectrum of the EuTc solution shows weak dependence with cholesterol addition

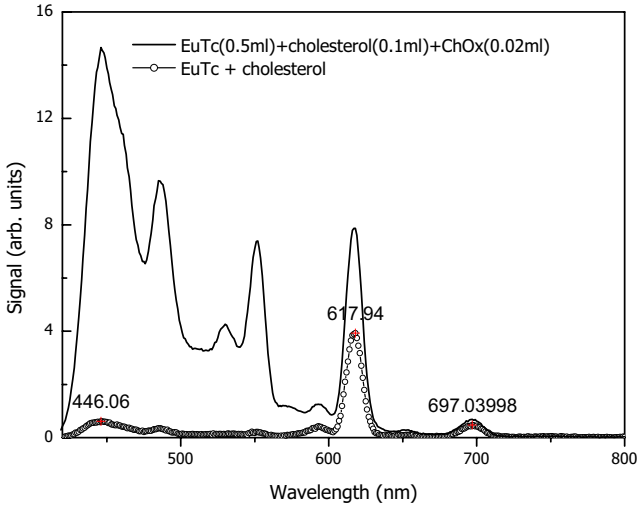

Fig. 2 Emission spectra of EuTc/cholesterol (*circles*) and EuTc/cholesterol in the presence of cholesterol oxidase (*line*) obtained under excitation at 405 nm

Figure 1 shows the absorption spectra for the pure EuTc and pure cholesterol solutions, along with solution V (x=10, 60, 100 and 200 μl) with 1, 6, 9 and 16% of cholesterol solutions. In this graph can be seen that pure cholesterol has no absorption in the range 300–500 nm, and that the pure EuTc has a large absorption band centered at 395 nm. This absorption band is caused by the presence of the tetracycline ligand which, in its uncomplexed form, has a slightly blue shifted absorption spectrum. The addition of cholesterol does not change significantly the shape or position of this band.

In Fig. 2, the emission spectra of EuTc + cholesterol (solution V) and EuTc+cholesterol+ChOx (cholesterol oxidase) obtained under excitation at 405 nm are shown. An enhancement of the 618 nm emission is observed. This enhancement is a direct consequence of the enzymatic oxidation reaction shown in Eq. 1. The H_2O_2 molecules produced substitute water molecules in the vicinity of europium ions, enhancing its emission. Since the quantity of H_2O_2 produced is proportional to the cholesterol quantity, the monitoring of the Eu emission enhancement can be used as a method to determine the cholesterol concentration.

$$\text{Cholesterol} + O_2 \Rightarrow \text{Cholesterone} + H_2O_2 \qquad (1)$$

Nevertheless, a simpler method, without the need of the enzymatic reaction is possible, and it is described here. In Fig. 3a the emission spectrum for all cholesterol concentrations of solution V are shown, and an enhancement of the EuTc emission with the cholesterol addition is observed, along with a small red shift of the emission. The emission enhancement shows that is possible to use EuTc to determine cholesterol concentrations without the use of the ChOx enzyme. In order to do this, a calibration curve was determined by fitting a linear function to the 618 nm emission intensity as a function of the cholesterol volume

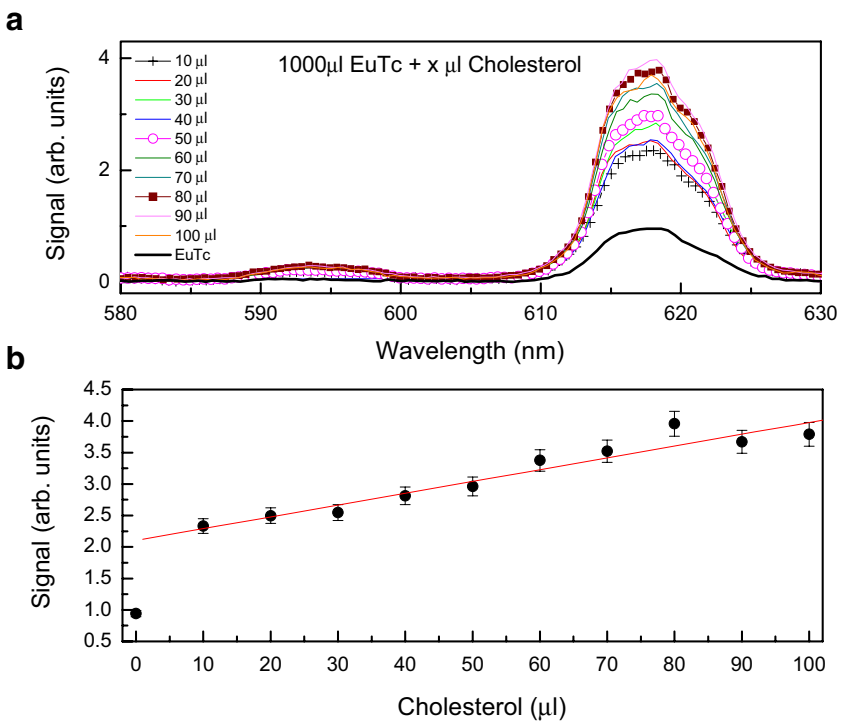

Fig. 3 Increase of the EuTc emission in the presence of cholesterol. **a** Emission spectra as a function of cholesterol concentration. **b** Dependence of the 618 nm emission peak intensity with the cholesterol concentration. The fitted linear function is used as a calibration curve

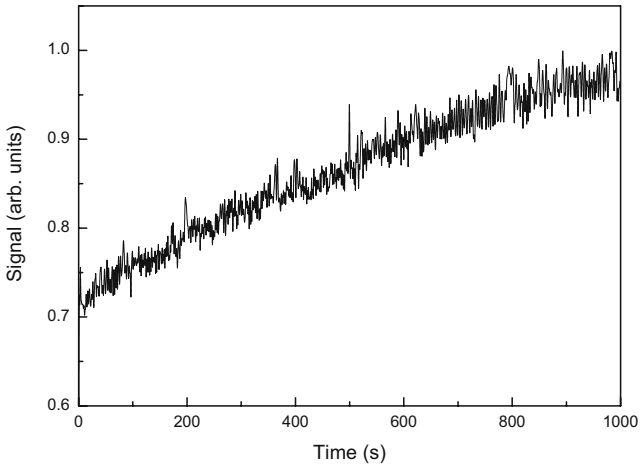

Fig. 4 Dependence of the of 618 nm emission intensity with the time showing that stabilization occurs after 1,000 s

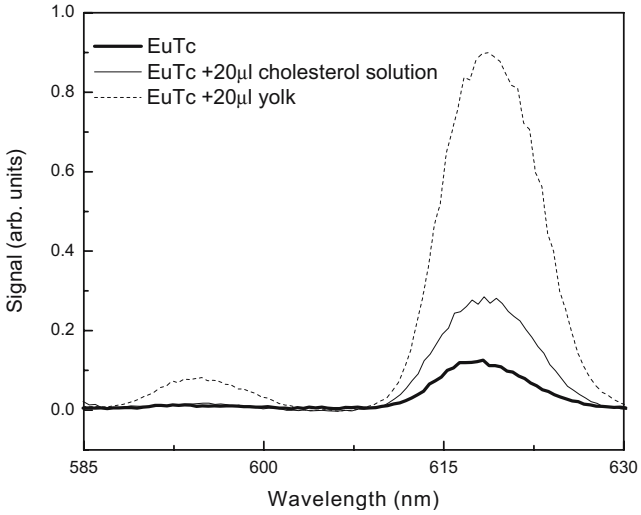

Fig. 6 Europium emission band in solutions of EuTc, EuTc with 20μl of cholesterol and EuTc with 20 μl of yolk egg obtained under excitation at 405 nm

(% of 200 mg/dl cholesterol solution), and the results are shown in Fig. 3b. This graphic shows that the emission intensity grows linearly with the cholesterol volume up to 90 μl of cholesterol (8.3% of cholesterol). The plot can be described by a linear equation of the type $y=A+Bx$, with (A= 2.3082±0.053, B=0.018±0.001 and r=0.9842). The limit of detection is 0.19 μl or 0.038 mg/dl (0.001 mM) cholesterol. Using sample volumes smaller than 10 μl is possible to measure cholesterol concentrations up to 800 mg/dl (~5.0 mM) without loss in the linearity. It is important to mention that the emission enhancement takes approximately 1,000 s to saturate, as shown in Fig. 4. This effect occurs probably due to the thermodynamic equilibrium and water displacement around europium ions due the cholesterol hydrophobic behavior. For the assay design is necessary to begin the measurements fifteen minutes after the sample preparation, but it is possible to measure the samples in the next morning or the next week without quality less in the results.

The $^5D_0 \rightarrow {}^7F_2$ europium transition lifetime for the solutions IV and V were measured under pulsed excitation at 405 nm, and the results are shown in Fig. 5a. It can be seen that the europium emission lifetime increases with the addition of cholesterol to the solution, rising from 35 μs for

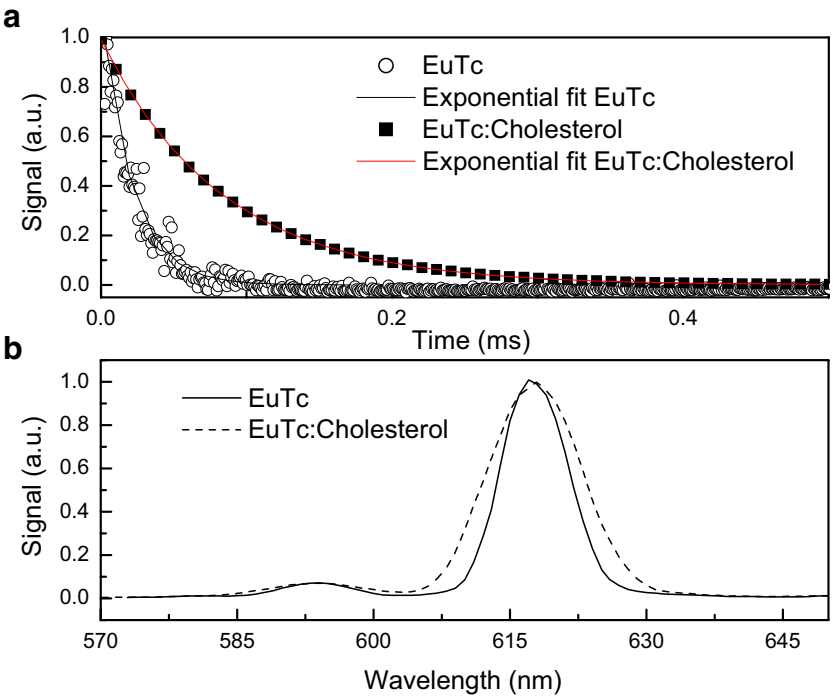

Fig. 5 **a** Normalized lifetime of EuTc and EuTc + cholesterol solutions (IV and V, respectively) obtained under excitation at 405 nm of a pulse Xenon lamp. **b** Normalized europium emission for solutions IV and V, showing the broadening in the solution containing cholesterol

the pure EuTc solution (IV) to 83 μs for the EuTc + cholesterol solution (V). Figure 5b shows that the width of europium emission band increases in the solution containing cholesterol.

To explain the emission intensity and lifetime enhancements, we suggest that water molecules in the solution EuTc/cholesterol are grouped around the polar hydroxyl groups of the cholesterol molecules, isolating europium ions connected to tetracycline molecules. Yet all the cholesterol molecules would arrange themselves so that the tiny polar hydroxyl groups were pointing into the water. With less water molecules in the vicinity of europium ions, the energy transfer to water molecules is minimized, and the energy is mainly kept in the Eu ions, increasing its lifetime and luminescence intensity, resulting in a luminescence quantum yield enhancement. The presence of cholesterol around europium results also in a broadening of europium emission band due to a multisite effect.

The steroid hormones, those that have molecular structures similar to the cholesterol molecule, should present similar spectroscopic behavior than cholesterol. Taking this into consideration we believe that the use of EuTc with enzymatic method is more useful for biological fluids once cholesterol oxidase will break only cholesterol molecules, and the luminescence increase will result only from the H_2O_2 produced in this reaction. In this case the calibration curve that must be used is the one mentioned in the papers that use the europium-tetracycline (EuTc) as sensors for hydrogen peroxide quantification (linearity between 2 and 400 μM hydrogen peroxide and 960 nM limit of detection) [21].

As an application of this simpler method, we estimated the cholesterol concentration in chicken egg yolk, which is known to have high cholesterol concentrations [22]. Figure 6 shows the emission spectra of solutions containing 20 μl of 200 mg/dl cholesterol pattern (solution V) and 20 μl of egg yolk added to 1 ml of EuTc (solution IV). In this graphic can be seen that the 618 nm emission intensity in the egg yolk spectrum is approximately four times higher than the peak intensity in solution V, indicating that the cholesterol concentration in the yolk should be around 800 mg/dl, if the linearity shown in Fig. 3b holds. It is important to remark that no research about other interferants in the yolk has been done and more effort will be considered in a future paper. It is known that alkali and earth alkaline ions have no effect in up to 100 mM concentrations. Transition metals as Co^{2+}, Fe^{3+} and Zn^{2+} quench in 0.1–1 mM concentrations, and Cu^{2+} is a particularly strong quencher even in <0.1 μM concentration. Ag+ increases luminescence intensity (<20 μM) and decreases emission more pronounced at concentrations exceeding 100 μM. Chloride, sulfate, acetate and nitrate are inert, but phosphate (>1 μM) gives an increase in intensity. Fluoride quenches significantly at >25 μM concentrations, bromide and iodide do not interfere up to 1 mM. Carbonate displays quenching at >200 μM. Among the organic species, citrate and malate interfere in giving an increase in the luminescence intensity of EuTc (and a decrease in the intensity of the EuTc in the presence of hydrogen peroxide) [23, 24].

This result shows the possibility of EuTc use on food sciences to determine cholesterol concentrations.

Calculation:

Pattern: 20 μl cholesterol solution (200mg/dl) + 1ml EuTc

Analyzed sample: 20 μl (analyzed sample) + 1ml EuTc

Cholesterol (mg/dl)=(Eu emission instensity of analyzed sample × 200 mg/dl)/ Eu emission intensity of pattern

Conclusions

An increase of the europium emission in europium-tetracycline complex solution was observed in the presence of pure cholesterol solution and cholesterol plus cholesterol oxidase solution, for the first time to our knowledge. This is the starting point for the determination of cholesterol concentrations in many solutions with a simple and low cost method that works in neutral pH.

The analyzed samples (EuTc + cholesterol) can be measured after a long storage time (several weeks) without loss of linearity of the calibration graph. Considering these facts we can prove that this effect is robust enough to be meaningful for the analysis of real world samples, like foods, calibrations solutions and biological fluids.

Once EuTc probe is commercially available to glucose analysis, an especial interest of this work is to analyze cholesterol and also steroid hormones as interferants that it can affect the results drastically.

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