Europium Tetracycline Biosensor for the Determination of Cholesterol

Lilia Coronato Courrol[a], Flávia Rodrigues de Oliveira Silva[b], Ricardo Elgul Samad[c], Ronaldo Domingues Mansano[b], Nilson Dias Vieira Júnior[c]
[a]Universidade Federal de São Paulo -UNIFESP – Campus Diadema – Rua Arthur Riedel, 275 – Eldorado - Diadema/SP- Brazil
[b]Universidade de São Paulo, Escola Politécnica, LSI, USP – São Paulo, SP
[c]Centro de Lasers e Aplicações, IPEN/CNEN-SP, São Paulo, SP –Brazil

ABSTRACT

Development of cholesterol biosensors is of great importance in clinical analysis because the concentration of cholesterol in blood is a fundamental parameter for the prevention and diagnosis of a number of clinical disorders such as heart disease, hypertension and arteriosclerosis. In general, determination of cholesterol is based on spectrophotometry; but this method involves complicated procedures and the cost is high because expensive enzyme must be used in each assay.

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 an easy-handling immobilization method with a cheap stable material. This method shows that the complex can be used as a sensor to determine cholesterol in biological systems with good selectivity, fast response, miniature size, and reproducible results.

Keywords: Cholesterol; Europium; Analytical analysis

1. INTRODUCTION

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 [1]. 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 component [2] 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 has two well-defined regions, a core and a surface layer [3]. The outer shell is composed by phospholipids, unesterified cholesterol and the apolipoprotein B-100. The inner core is composed mainly by cholesteryl esters and triglycerides [4]. Often, when doctors talk to their patients about the health concerns of cholesterol, they are referring to the LDL, popularly known as "bad cholesterol". "Good cholesterol" is high-density lipoprotein (HDL). 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 [5, 6]. According to the American Heart Association, normal levels of the sanguineous total cholesterol are below of 200mg/dl, while concentrations above of 240mg/dl are high risk factors for coronaries illnesses [7]. For these reasons, cholesterol has

Advanced Biomedical and Clinical Diagnostic Systems V
edited by Tuan Vo-Dinh, Warren S. Grundfest, David A. Benaron, Gerald E. Cohn, Ramesh Raghavachari
Proc. of SPIE Vol. 6430, 643017, (2007) · 1605-7422/07/$18 · doi: 10.1117/12.697956

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 [8, 9, 10, 11 , 12].

When in aqueous solution, lanthanide ions exhibits weak luminescence due to its small absorption cross section and strong energy transfer to surrounding water molecules [13, 14]. 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 [15] that transfers the absorbed energy to the lanthanide through an intramolecular process [16], 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 [17], 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 [18], 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 this molecule capability of displacing water molecules from the Eu neighborhood [19]. These characteristics make the complex highly sensitive and specific for H_2O_2 detection [20]. As an additional advantage, the EuTc complex can be excited by commercial LEDs and lasers, and works in neutral pH [21].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.

2. 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 $mmolL^{-1}$ 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; 200mg/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: EuTc solution – Mix 10 ml of solution II and 10 ml of solution III and 80 ml solution I.

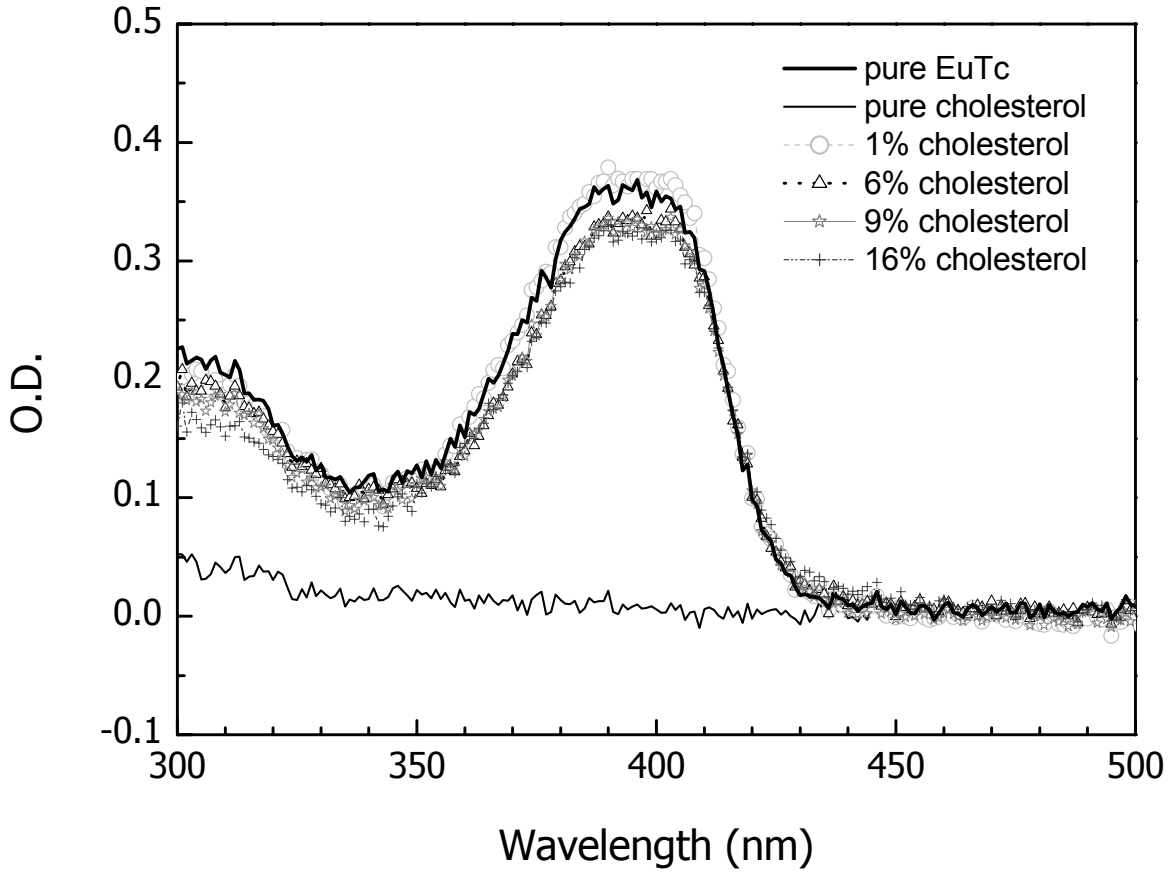
Solution V: EuTc-cholesterol solutions – Mix of 1 ml of solution IV with *x* μL (*x*=10, 20, 30, 40, 50, 60, 70, 80, 90, 100) of cholesterol solution (200mg/dL).

The absorption spectra of all samples were measured in the range 200 nm-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 0.5m 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 fewer than 5%.

3. RESULTS

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

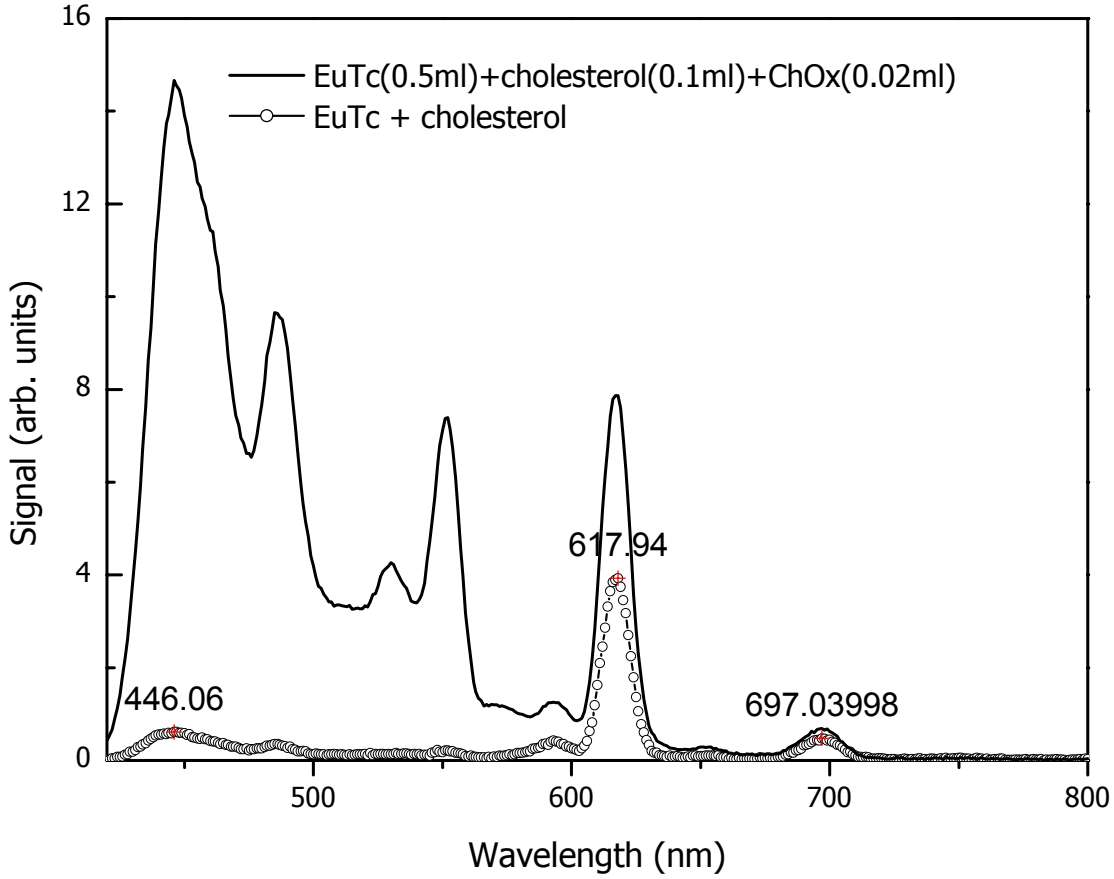
Error! Reference source not found. shows the absorption spectra for the pure EuTc and pure cholesterol solutions, along with solution V (*x*=10, 60, 100 and 200 μl) with 1%, 6%, 9% and 16% of cholesterol solutions. In this graph can be seen that pure cholesterol has no absorption in the range 300 nm-500 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 significatively the shape or position of this band.



Error! Reference source not found.. Absorption spectra for cholesterol, EuTc and EuTc+cholesterol solutions. The absorption spectra of the EuTc solution shows weak dependence with cholesterol addition

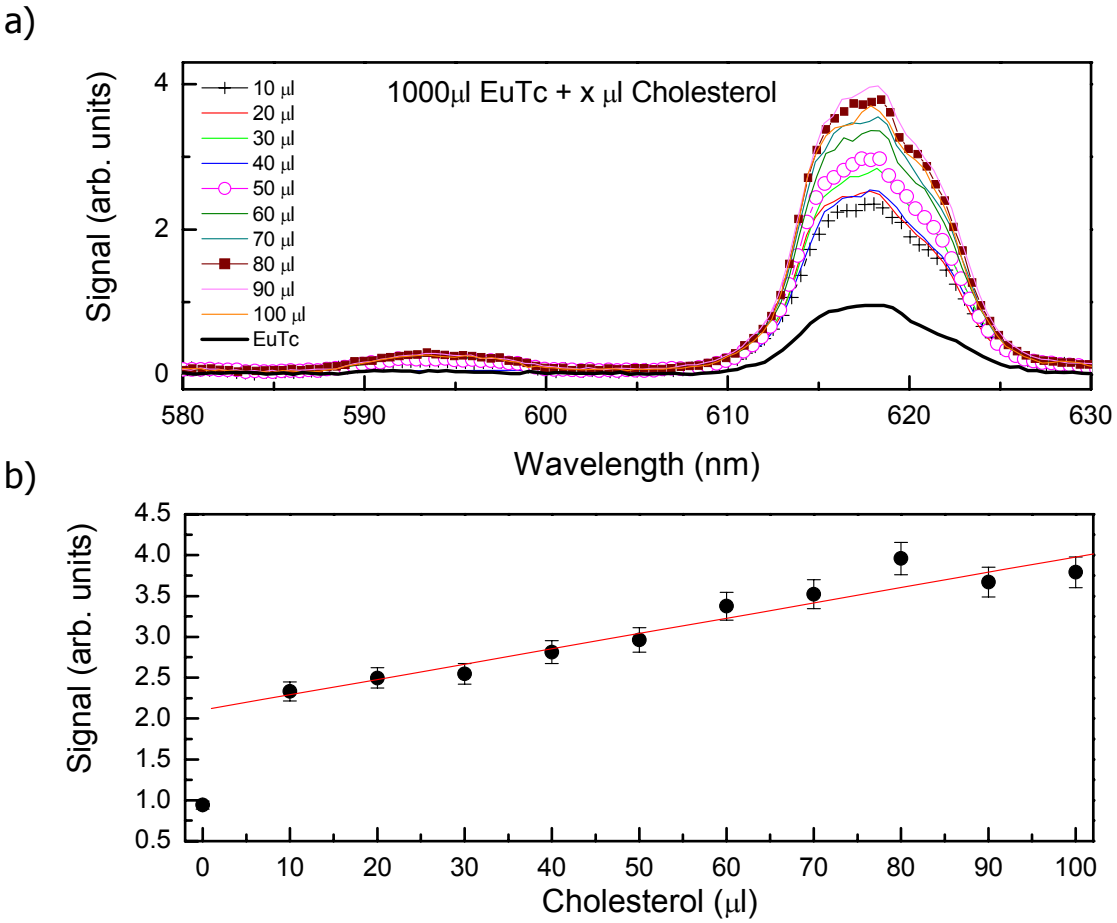
In **Error! Reference source not found.**, the emission spectra of EuTc+cholesterol (solution V) and EuTc+cholesterol+ChOx (cholesterol oxidase) obtained under excitation at 405 nm is shown. An enhancement of the 618 nm emission is observed. This enhancement is a direct consequence of the enzymatic oxidation reaction schematized in equation 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.

$$\text{Cholesterol} + O_2 \xrightarrow{\text{ChOx}} \text{Cholesterone} + H_2O_2 \quad \textbf{(1)}$$

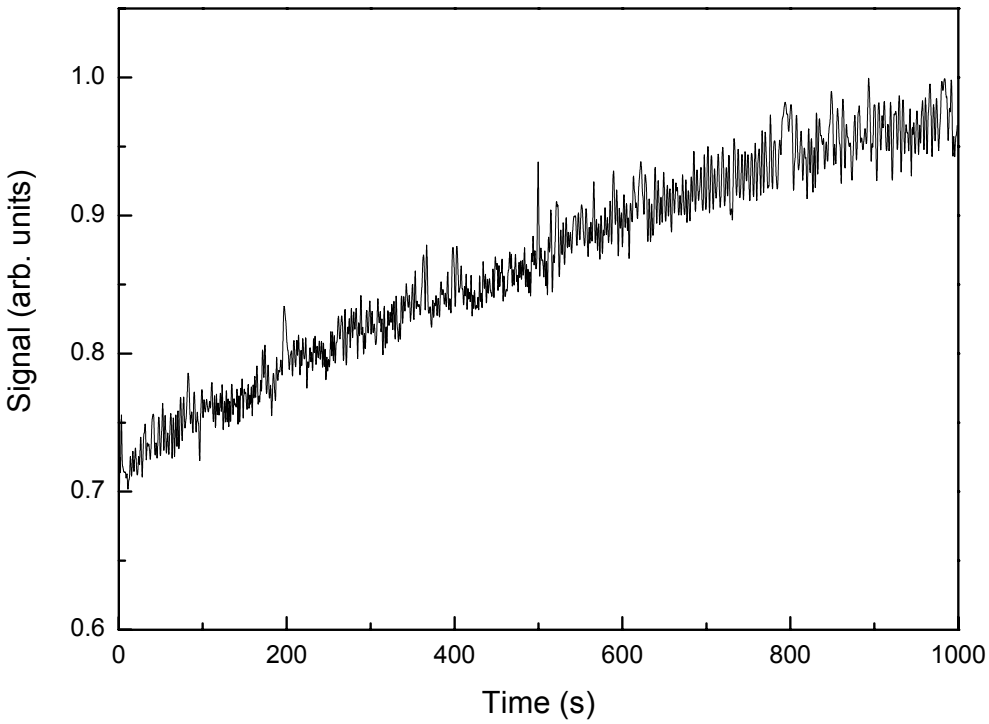


Error! Reference source not found.. Emission spectra of EuTc:Cholesterol (circles) and EuTc:Cholesterol in the presence of cholesterol oxidase (line) obtained under excitation at 405 nm.

Nevertheless, a simpler method, without the need of the enzymatic reaction is possible, and it is described here. In **Error! Reference source not found.**a the emission spectra 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 obtained by fitting a function to the 618 nm emission intensity as a function of the cholesterol concentration, and the results are shown in **Error! Reference source not found.**b. In this graphic it can be seen that the intensity grows linearly with the cholesterol concentration in the range up to 90 µl of cholesterol (8.3% of cholesterol). It is important to mention that the emission enhancement is not instantaneous, taking approximately 1000 s to saturate, as can be seen in figure 4.

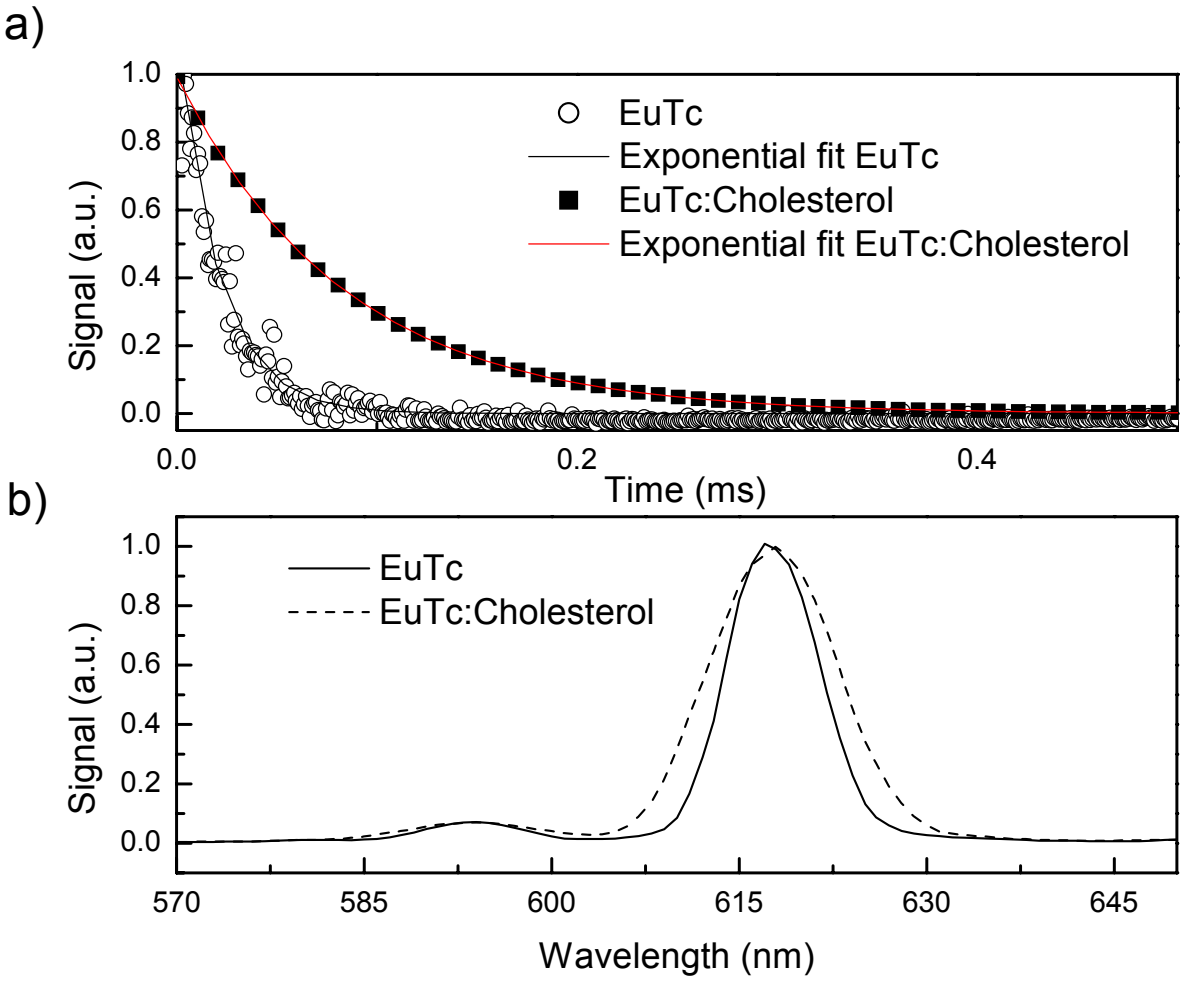


Error! Reference source not found.. Increase of the EuTc emission in the presence of cholesterol. a) Emission spectra as a function of cholesterol concentration. b) Dependence of the 618 nm emission peak intensity with the cholesterol concentration. The fitted linear function is used as a calibration curve.



Error! Reference source not found.. Dependence of the of 618 nm emission intensity with the time showing that stabilization occurs after 1000 s.

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 **Error! Reference source not found.**a. It can be seen that the europium emission lifetime increases with the addition of cholesterol to the solution, rising from 35 μs for the pure EuTc solution (IV) to 83 μs for the EuTc+cholesterol solution (V). **Error! Reference source not found.**b shows that the width of europium emission band increases in the solution containing cholesterol.



Error! Reference source not found.. 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.

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.

4. 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. The intensity grows linearly with the cholesterol concentration in the range up to 90 μl of cholesterol (8.3% of cholesterol). The europium emission lifetime increases with the addition of cholesterol to the solution, rising from 35 μs for the pure EuTc solution to 83 μs for the EuTc+cholesterol solution.

This is the starting point for the determination of the cholesterol concentrations in many solutions, including blood, with a simple low cost method. Another factor that favors this determination is that the pH of the EuTc complex is ~7, next to the sanguineous pH.

REFERENCES

1. National Cholesterol Education Program. Second report of the expert panel on detection, evaluation, and treatment of high blood cholesterol in adults. NIH Pub. No. 93-3095. Bethesda, MD: National Heart, Lung, and Blood Institute, (1993).
2. Harvey et al., Biochemistry: 3rd Edition, Baltimore: Lippincott, pp. 235-238, (2005).
3. S.L. Gómez, R.F. Turchiello, M.C. Jurado, P. Boschcov, M. Gidlund and A.M. Figueiredo Neto, Chemistry and Physics of Lipids, Volume 132, Issue 2, , Pages 185-195, (2004).
4. T. Hevonoja, M.O. Pentikäinen, M.T. Hyvönen, P.T. Kovanen and M. Ala-Korpela, Biochim. Biophys. Acta 1488 189–210, (2000).
5. Castelli W. Value Health. 1(2):105-9, (1998).
6. Danese C, Vestri AR, D'Alfonso V, Deriu G, Dispensa S, Baldini R, Ambrosino M, Colotto M., Clin Ter. 157(1):9-13, (2006).
7. Detection and Evaluation, Circulation, 106: 3227-3234, (2002).
8. Jabbar J, Siddiqui I, Raza Q., J Pak Med Assoc. 56(2):59-61, (2006).
9. Vega GL, Grundy SM. Does measurement of apolipoprotein B have a place in cholesterol management, *Arteriosclerosis*; 10:668 -71, (1990).
10. Danese C, Vestri AR, D'Alfonso V, Deriu G, Dispensa S, Baldini R, Ambrosino M, Colotto M., Clin Ter.,157(1):9-13, (2006).
11. W. Trettnak,O.S. Wolfbeis, Analytical Biochemistry,184, 1 , 124-127**,** (1990).
12. Anjan Kumar Basu, Parimal Chattopadhyay, Utpal Roychoudhuri and Runu Chakraborty Bioelectrochemistry, In Press, Corrected Proof, Available online, (2006).
13. Arnaud N., Georges J., Spectrochim Acta A Mol Biomol Spectrosc., 1829-1840, (2003).
14. P. R. Selvin, in Applied Fluorescence in Chemistry, Biology and Medicine, edited by W. Rettig, B. Stremel, S. Schrader, Springer Verlag, pp. 457-487, (1999).
15. J. M. Lehn, Angew. Chem. Int. Ed. Engl. 29, 1304, (1990).
16. Selvin PR, Hearst JE. Proc Natl Acad Sci U S A. Oct 11;91(21):10024-8, (1994) .
17. J. Magnam, D. Barthes, J.J. Giraud., Ann Pharm Fr.,;42(2):155-9, (1984).
18. Flávia Rodrigues da Silva, Lilia Coronato Courrol, Luis Vicente Gomes Tarelho, Laércio Gomes, Nilson Dias Vieira Júnior, Journal of Fluorescence, v. 15, n. 5, p. 667-671, (2005).
19. Y. Rakicioglu, J. H. Perrin, S. G. Schulman, Journal of Pharm. And Biom. Aanlysis, 20, 397-399, (1999).
20. Lin Z, Wu M, Wolfbeis OS, Schaeferling M., Chemistry, Mar 20;12(10):2730-8, (2006).
21. M. Wu, Z. Lin, M. Schaeferling, A. Durkop, O.S. Wolfbeis, Anal biochempp., 340, 66-73, (2004).