J Fluoresc (2011) 21:887–892
DOI 10.1007/s10895-010-0602-8

ORIGINAL PAPER

Investigation of the Europium Emission Spectra of the Europium-Oxytetracycline Complex in the Presence of Human Low-Density Lipoproteins

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Received: 29 October 2009 / Accepted: 29 January 2010 / Published online: 23 February 2010

Abstract Low-Density Lipoprotein (LDL), often known as "bad cholesterol" is one of the responsible to increase the risk of coronary arterial diseases. For this reason, the cholesterol present in the LDL particle has become one of the main parameters to be quantified in routine clinical diagnosis. A number of tools are available to assess LDL particles and estimate the cholesterol concentration in the blood. The most common methods to quantify the LDL in the plasma are the density gradient ultracentrifugation and nuclear magnetic resonance (NMR). However, these techniques require special equipments and can take a long time to provide the results. In this paper, we report on the increase of the Europium emission in Europium-oxytetracycline complex aqueous solutions in the presence of LDL. This increase is proportional to the LDL concentration in the solution. This phenomenum can be used to develop a method to quantify the number of LDL particles in a sample. A comparison between the performances of the oxytetracycline and the tetracycline in the complexes is also made.

Keywords LDL · Europium · Fluorescence · Biosensor

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Introduction

Tetracycline (Tc) and oxytetracycline (OTc) are antibiotics of the tetracycline group, which present a wide range of antimicrobial activity against Gram-positive and Gram-negative bacteria [1-4]. When the Europium forms a complex with tetracyclines [1, 5-10], the ion luminescence can be observed due to the ligand large absorption and an antenna-effect [7] that transfers the absorbed energy to the Europium through an intramolecular process. The ion luminescence is also enhanced by the isolation that the ligand provides from the surrounding water molecules, preventing energy transfer to them. The Europium-tetracycline complex (EuTc) has an absorption band centered around 400 nm, emission band around 615 nm and a large Stokes-Shift. This luminescence was observed to increase up to 15 times in the presence of H_2O_2 due to this molecule capability of remove water molecules from the Eu neighborhood [11, 12]. These characteristics make the complex highly sensitive and specific for H_2O_2 detection [12]. In previous works, it was demonstrated that the Europium luminescence of Europium Tetracycline complex increases in the presence of Low-Density Lipoprotein (LDL) [7, 13-15].

Cholesterol is transported by plasma lipoproteins, low-density lipoprotein (LDL) often known as "bad cholesterol", is the main carry cholesterol with about two-thirds of the circulating cholesterol in humans. The structure of the LDL particles has two well-defined regions, a core and a surface layer [16]. The outer shell is composed of phospholipids, unesterified cholesterol and the apolipoprotein B-100. The inner core is mainly composed of cholesteryl esters and triglycerides [17]. Is well-established that modifications in the structure of LDL are currently to be prerequisite for initiation of intimal lipid acumulation [18]. Thus, levels cholesterol and associated lipids in plasma are important predictive tools utilized clinically to determine risk of coronary arterial disease (CAD). According to the American Heart Association, normal levels of the cholesterol are below 200 mg/dL, while concentrations above of 240 mg/dL indicate high risk to develop. A number of tools are available to quantify LDL particles in plasma, e.g., the density gradient ultracentrifugation [19-22], nondenaturing gradient gel electrophoresis (NDGGE) [23], and nuclear magnetic resonance (NMR) spectroscopy [24]. These procedures have the disadvantages of being labor-intensive, technically demanding, expensive, and time demanding [25]. In a previous work we demonstrated the enhancement of Europium-tetracycline complex emission in the presence of LDL solutions [13]. In this paper we describe an optical method for LDL quantification, based on the enhancement of the oxytetracycline Europium complex emission. A comparison between the use of tetracycline and oxytetracycline in the LDL quantification is also made. To allow this comparison, the protocol used in the present study was applied to both Tc and OTc with human LDL. The presence of some metallic species in the samples, sometimes present in the plasma, was also investigated.

Materials and methods

Isolation of LDL

Blood was drawn from healthy fasting (12 h) normolipidemic blood-donor volunteers, and plasma was obtained after centrifugation at 1,000 g and at 4°C, during 15 min. Thereafter benzamidine (2 mM), gentamicin (0.5%), chloramphenicol (0.25%), PMSF (phenyl-methyl-sulfonyl-fluoride) (0.5 mM), and aprotinin (0.1 unit/mL) were added to the samples. LDL was isolated by sequential ultracentrifugation at 100,000 g, at 4 °C, using a 75 Ti rotor (Beckman Instruments), and after that dialyzed at 4 °C against PBS (phosphate buffered saline) in pH 7.4, with 0.01% EDTA (ethylenediaminetetraacetic acid). The LDL was sterilized via filtration through a 0.22 μm-pore filter (Milipore—German). The lipoprotein concentration was quantified using the BCA kit (PIERCE) using bovine serum (BSA) as standard.

Europium tetracycline and oxitetracycline solutions

The Europium tetracycline (EuTc) and Europium oxytetracycline (EuOTc) complexes solutions were prepared starting from the 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 and oxytetracycline hydrochlorides used were purchased from Sigma-Aldrich. The prepared solutions were:

Solution I: Buffer solution: 2.09 g of MOPS in 1000 mL distilled water (with pH adjusted to 6.9).

Solution II: Different molar ratio Europium solutions:

- 21 μmol·L^{-1} Eu^{3+} solution; 0.0079 g of $EuCl_3 \cdot 6H_2O$ in 10 mL solution I.
- 31,5 μmol·L^{-1} Eu^{3+} solution; 0.0115 g of $EuCl_3 \cdot 6H_2O$ in 10 mL solution I.
- 42 μmol·L^{-1} Eu^{3+} solution; 0.0154 g of $EuCl_3 \cdot 6H_2O$ in 10 mL solution I.
- 52,5 μmol·L^{-1} Eu^{3+} solution; 0.0192 g of $EuCl_3 \cdot 6H_2O$ in 10 mL solution I.
- 63 μmol·L^{-1} Eu^{3+} solution; 0.0231 g of $EuCl_3 \cdot 6H_2O$ in 10 mL solution I.
- 73,5 μmol·L^{-1} Eu^{3+} solution; 0.0269 g of $EuCl_3 \cdot 6H_2O$ in 10 mL solution I.
- 84 μmol·L^{-1} Eu^{3+} solution; 0.0307 g of $EuCl_3 \cdot 6H_2O$ in 10 mL solution I.

Solution III: 21 μmol·L^{-1} of Tetracycline/Oxytetracycline (thereafter this label means Tc or OTc) in 10 mL of solution I.

Solution IV: EuTc/EuOTc: Mix of 10 mL Solution II with 10 mL Solution III and MOPS to complete 100 mL.

Solution V: EuTc/EuOTc-LDL: 1 mL of solution IV and 10 μL of LDL.

Optical characterization

The absorption spectra of all the samples were measured at room temperature in the range 200 nm–500 nm, using a Varian Cary 17D Spectrometer. The emission spectra were obtained by exciting the samples placed inside a 1 mm optical path cuvette. The emission spectra of the samples were analyzed with a Fluorolog 3, from Horiba Jobin Yvon,.

To investigate the effect in the emission spectra of the EuOTc of the presence of some metallic species in the

samples, aqueous standards of Cu, Co, Mn ,Zn, Mg, Fe, Ca, Ni, Al and Ag, at concentrations normally found in plasma, (Table 1) were introduced in the solutions.

Results

Figure 1 shows the optical absorption of OTc and EuOTc solutions prepared in MOPS buffer at pH=6.9 (solutions I, II, III and IV). The OTc solution presents absorption bands at 247, 275 and 357 nm. In the presence of the Eu^{3+} ion, a red shift is observed for the third band and a blue shift for the first. Figure 2 shows the absorption spectra of EuOTc (1:1) and EuTc (3:1) complexes. These complexes present absorption peaks at 390 nm and 400 nm, respectively.

Figure 3a shows the emission spectra of Europium and OTc solutions, excited with light of wavelengths 400 nm and 357 nm, respectively. We observe that the OTc solution has an emission band centered around 480 nm and the Europium solution presents the characteristic europium emission bands due the transitions $^5D_0 \rightarrow {}^7F_J$ (J=0, 1, 2, 3, 4) and $^5D_1 \rightarrow {}^7F_J$ (J=1, 2, 3, 5, 6). Figure 3b shows the emission spectra of the EuOTc complexes, excited at 400 nm. In this case, the OTc molecule acts as an antenna, absorbing the excitation light and transferring the energy to the Europium ion. Comparing the emission spectra of complexes prepared with different molar ratios, we observe that the molar ratio 1:1 emits the higher intensity, and for this reason, it was chosen for the subsequent experiments.

Figure 4 shows a comparison between the EuOTc (1:1) and the EuTc (3:1) complexes solutions in the presence of 1.558 mg/L of LDL. Samples were excited at 390 nm and 400 nm, respectively, and an about six times increase was observed in the emission intensity peak of EuOTc complex when compared with the EuTc complex.

Table 1 List of the studied metallic species and their concentrations in the solution

Metallic specie	Concentration
n^{2+}	20 μM
Al^{3+}	12 μM
Ca^{2+}	1.12 μM
Co^{2+}	0.1 μM
Cu^{2+}	11.02 μM
Fe^{3+}	10.74 μM
Mg^{2+}	0.75 mM–1 mM
Mn^{2+}	0.2 μM
Ni^{2+}	0.1 μM
Ag^{+}	0.093 μM

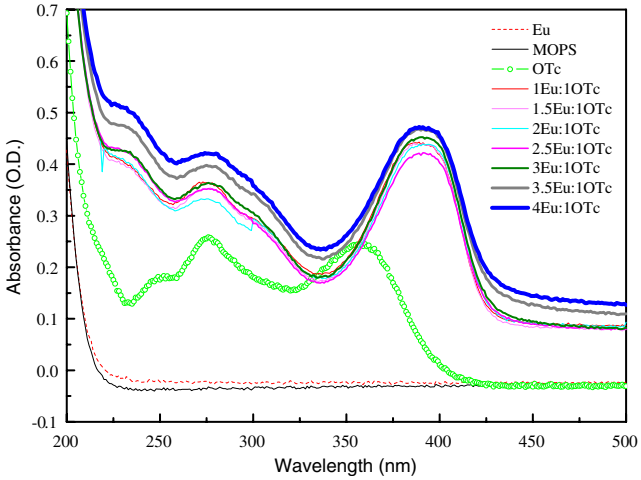

Fig. 1 Absorption spectra of Europium solution (*dash curve*), MOPS solution (*thin continuous line*), oxytetracycline solution (*white circles*) and EuOTc solutions with different molar ratios: 1Eu:1OT; 1.5Eu:1OTc; 2Eu: 1OTc; 2.5Eu:11OTc; 3Eu: 1OTc; 3.5Eu:1Ox; 4Eu: 1OTc (*thick continuous lines*)

Figures 5 and 6 show the experimental points obtained calculating the area below the emission spectra of the EuTc and EuOTc complexes in the presence of different LDL concentrations. These plots are well described by linear functions $Y = A + BX$, where X is the LDL concentration. The parameters (A and B) of the linear functions for EuTc and EuOTc obtained are: (1.73×10^7 and 1.55 10^7 mL/mg) and (4.98×10^7 and 2.80×10^6 mL/mg), respectively. With these values it was possible to calculate the LOD (limit of detection) and the SD (standard deviation) in the case of both the EuTc and EuOTc: (0.33 mg/mL and 1.70×10^6) and (0.22 mg/mL and 2.01×10^6), respectively. These results indicate that the use of EuOTc in our method to determine the LDL concentration in a sample improved the LOD with respect to the EuTc complex.

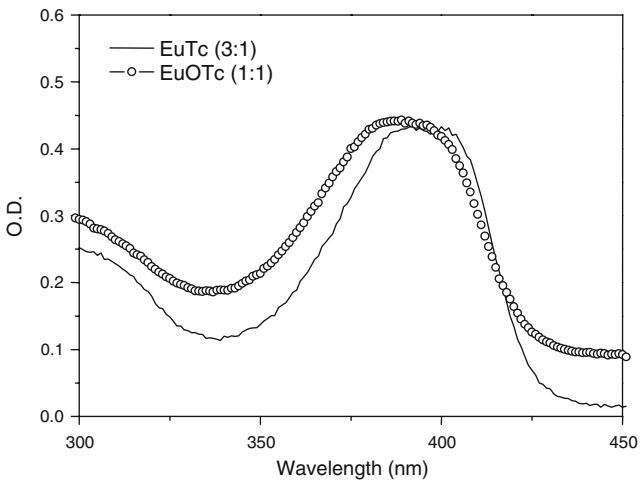

Fig. 2 Absorption spectra of EuTc (3:1) and EuOTc (1:1)

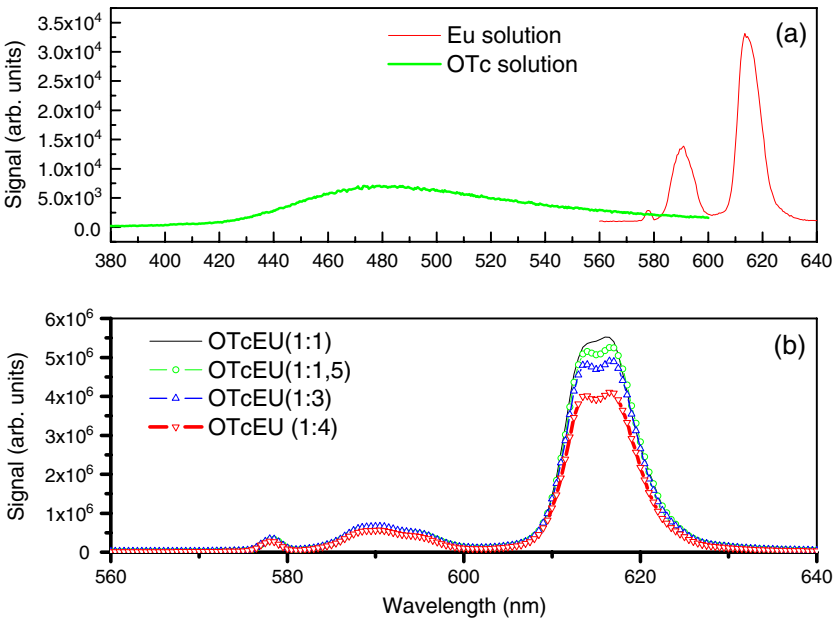

Fig. 3 **a** Europium emission excited at 400 nm and oxytetracicline emission band excited 357 nm e **b** Europium emission band for different molar ratios of EuOTc complex

Figure 7 shows the effect of the presence of some metallic species in the solutions. Traces of these species may be encountered in the human plasma. The data presented represent the peak intensity of the emission spectra of each sample, normalized with respect to the peak intensity of the EuOTc solution. We observe the decrease in the luminescence intensity of the EuOTc complex in aqueous solution in the presence of Cu. The presence of Co, Mn and Ni increases the emission intensity. The presence of the other studied metallic species has little effect on the EuOTc luminescence. Zhu and co-workers [26] showed that the presence of glucose, tryptophan and glycine in the EuTc complexes slightly modifies the Europium emission spectra. We could expect the same behavior for the EuOTc complex.

Figure 8 shows the Europium emission lifetime in the EuTc (Fig. 8a) and EuOTc (Fig. 8b) solutions, with and without the presence of LDL. Generally, complex systems have multiple fluorescent species, and hence, the fluorescence intensity decay cannot be fitted by a single exponential function. These are the cases of EuTc and EuOTc complexes, with and without the LDL. A multi-exponential function is needed to describe the experimental data. In the present cases, the experimental data can be fitted by a two-exponential function:

$$y = y_0 + A_1 e^{(-t/\tau_1)} + A_2 e^{(-t/\tau_2)} \quad (1)$$

where A_i and τ_i are the i^{th} pre-exponential factor (amplitude) and the lifetime in the multi-exponential decay,

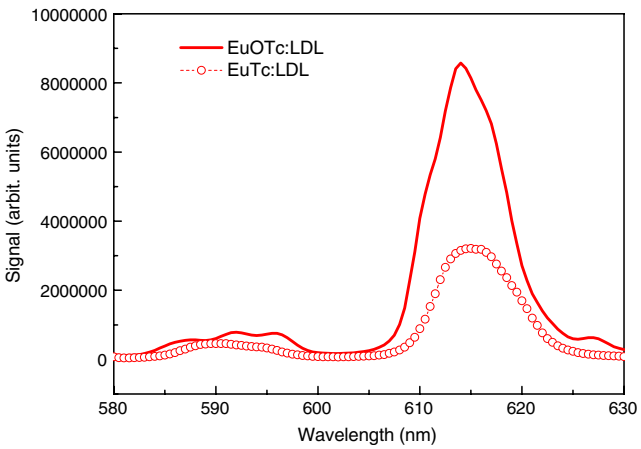

Fig. 4 Emission spectra of EuTc (3:1) and EuOTc (1:1) with LDL (1.558 mg/mL)

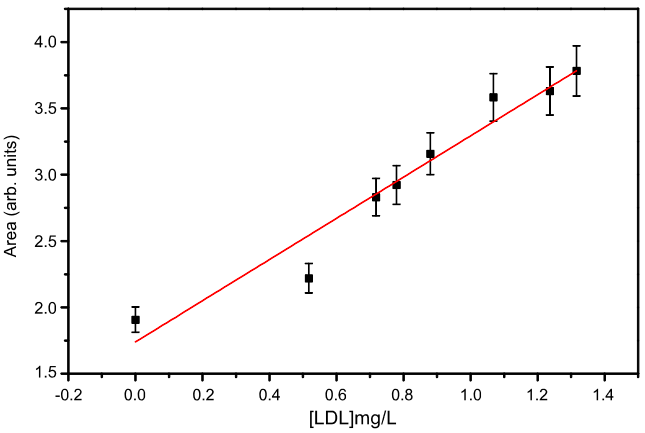

Fig. 5 EuTc:LDL calibration curve. Integrated area of the emission spectra as a function of the LDL concentration. The solid line corresponds to a linear fit to the experimental data

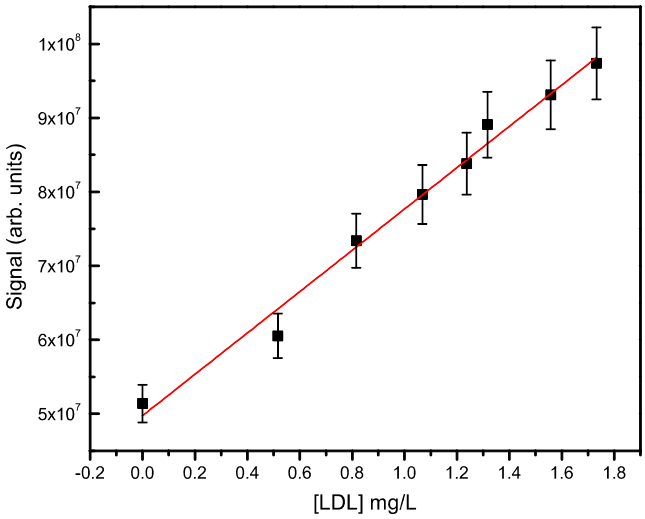

Fig. 6 EuOTc:LDL calibration curve. Integrated area of the emission spectra as a function of the LDL concentration. The solid line corresponds to a linear fit to the experimental data

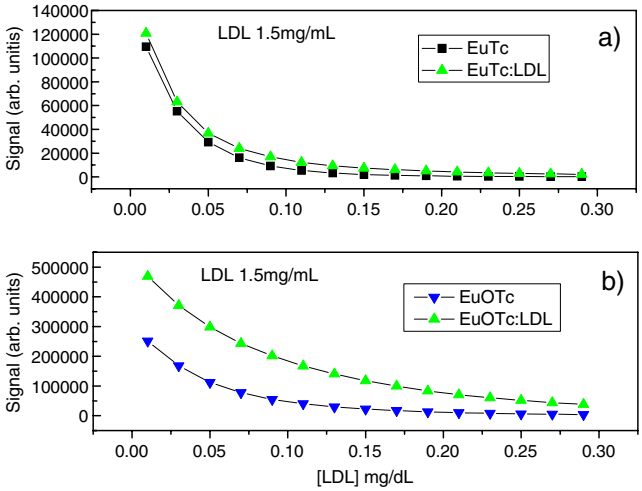

Fig. 8 Europium emission lifetime measurement. **a** EuTc with and without the presence of LDL; **b** EuOTc with and without the presence of LDL

respectively. In the case of multi-exponential decays the average lifetime, which is proportional to the total area under the fluorescence decay curve, is defined by:

$$\tau_{av} = \frac{\sum_i A_i \tau_i^2}{\sum_i A_i \tau_i} \quad (2)$$

The average lifetimes of the samples obtained by fitting Eq. 1 to the decay curves are~33 μs and 50 μs for the EuTc and EuOTc without LDL, respectively, and 88 μs and 110 μs for the EuTc and EuOTc with LDL (1.5 mg/mL), respectively. These results show that the fluorescence of the EuOTc:LDL systems is less quenched by molecular oxygen when compared with the EuTc:LDL complex.

To explain the Europium emission enhancement of both the EuTc and EuOTc in the presence of LDL, we suggest that water molecules in the solution containing LDL are grouped around the polar hydroxyl groups of the LDL particles, isolating Europium ions connected to tetracycline molecules. As the surfaces of the LDL particles have the head groups of the phospholipids, together with the APO-B 100 protein, water molecules from the solution form a hydration layer around the particles. 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, enhancing the luminescence quantum yield.

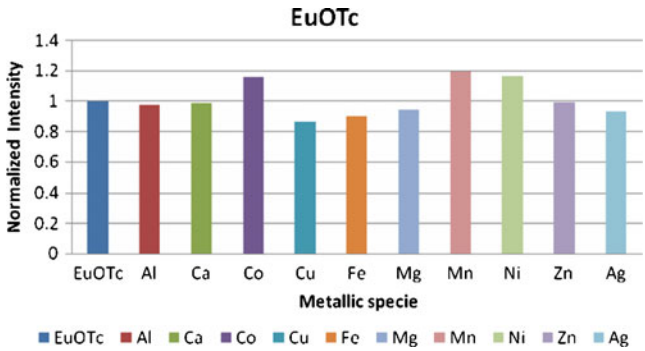

Fig. 7 Peak intensity of the emission spectra of the EuOTc sample in the presence of some metallic traces, normalized with respect to the peak intensity of the pure EuOTc solution

Conclusions

A characteristic increase of the Europium emission in EuOTc complex aqueous solution was observed in presence of different concentrations of LDL. We verified that the EuOTc complex emission intensity is bigger than that of the EuTc complex. The analysis of the limits of detection in our method to determine the LDL concentration in a sample with both EuTc and EuOTc complexes showed that the use of EuOTc is better than that of EuTc. Both complexes, however, are appropriate in the quantification of LDL.

Acknowledgments The authors thank CNPq, FAPESP and INCT-FCx (Instituto Nacional de Ciências e Tecnologia de Fluidos Complexos) for finantial support.

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